Beiträge zur Tabakforschung International/*Contributions to Tobacco Research* Volume 25 · No. 3 · August 2012

DOI: 10.2478/cttr-2013-0921

Influence of Additives on Cigarette Related Health Risks *

by

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*Received: 27th July 2011 – accepted: 25th July 2012

SUMMARY

Tobacco additives play an important role in the manufacturing and for the quality of tobacco products, particularly cigarettes and roll-your-own tobaccos. Attention is increasingly given to the potential effects of additives on consumer behavior and health. This review is intended to compile, collate and - to some degree - evaluate the wealth of pertinent scientific information available from the published literature and other special sources. At first, the reasons are set forth for the use of additives in cigarette manufacturing. In response to the growing controversy over the attractiveness and addictiveness of smoking, the clarification of terms and concepts is followed by a detailed discussion of two kinds of substances with particular relevance: Additives like ammonium compounds that are claimed to increase nicotine availability, and additives that are claimed to increase nicotine addictiveness.

The composition and toxicity of mainstream smoke of cigarettes with and without additives are assessed in several respects. The potentials of pyrolysis studies are explored by looking at a number of key studies and some basic considerations regarding *in vitro* and *in vivo* toxicity testing are addressed. Five major literature reviews on additives published between 1994 and 2004, and the results of several comprehensive experimental studies covering a large range of additives, released between 2002 and 2012, are dealt with in detail. Single tobacco additives of particular importance (menthol, glycerol, 1,2-propylene glycol, sorbitol, sugars, cocoa, licorice, citric acid, triacetin, and ammonium compounds) are discussed in dedicated chapters, which are generally subdivided into special sections: Use and toxicological assessment; inclusion level in cigarettes, transfer and pyrolysis; attractiveness and addictiveness; effect on cigarette mainstream smoke composition; effect on cigarette mainstream smoke toxicity. Epidemiological findings and data obtained by the biomonitoring of smokers consuming cigarettes with and without additives are compiled and interpreted specifically for American blend cigarettes, Virginia cigarettes, “French” (dark) cigarettes and menthol cigarettes whereby the focus is on the effects of additives on smoking topography and potential health risks.

Opinionated reviews were published in recent years that are compromised by arbitrary selection of sources and unbalanced views. Leaving those unconsidered, the aggregated scientific knowledge shows that tobacco additives have only occasional and limited effects on cigarette mainstream smoke composition, which are almost never reflected in the results of toxicological *in vitro* assays or *in vivo* studies. This supports the conclusion that tobacco additives are not likely to increase the known health risks of smoking. There is also no evidence for sustaining claims that certain additives increase nicotine availability or nicotine addictiveness. [Beitr. Tabakforsch. Int. 25 (2012) 411–493]

ZUSAMMENFASSUNG

Zusatzstoffe im Tabak spielen eine wichtige Rolle bei der Herstellung und für die Qualität von Tabakprodukten, insbesondere von Zigaretten und roll-your-own Tabaken. Zunehmend richtet sich die Aufmerksamkeit auf die möglichen Auswirkungen von Zusatzstoffen auf das Konsumverhalten und die Gesundheit der Konsumenten. Diese Übersichtsarbeit hat das Ziel, die Fülle an einschlägiger wissenschaftlicher Information, die in Veröffentlichungen und aus anderen speziellen Quellen verfügbar ist, zu sammeln, zu ordnen und - bis zu einem gewissen Grade - zu bewerten. Zunächst werden die Gründe für den Einsatz von Zusatzstoffen bei der Zigarettenherstellung dargelegt. Als Beitrag zu der intensiver werdenden Kontroverse über die Attraktivität und das Suchtpotential des Rauchens werden Begriffe und Konzepte erläutert, ergänzt durch die detaillierte Diskussion von zwei besonders relevanten Substanzgruppen. Dabei handelt es sich um Zusatzstoffe wie Ammoniumverbindungen, von denen behauptet wird, sie würden die Verfügbarkeit von Nikotin erhöhen, und Zusatzstoffe, bei denen diskutiert wird, sie würden das Suchtpotential von Nikotin verstärken.

Die Zusammensetzung und Toxizität des Hauptstromrauchs von Zigaretten mit und ohne Zusatzstoffe werden unter mehreren Gesichtspunkten beurteilt. Der Wert von Pyrolysestudien wird durch die Betrachtung einer Reihe von Schlüsselstudien ausgeleuchtet, und einige grundsätzliche Überlegungen zu *in vitro* und *in vivo* Toxizitätsprüfungen werden besprochen. Im Einzelnen diskutiert werden fünf größere Literaturübersichten zu Zusatzstoffen, die zwischen 1994 und 2004 publiziert wurden, sowie die Ergebnisse mehrerer experimenteller, zwischen 2002 und 2012 veröffentlichter Studien, die einen großen Bereich von Zusatzstoffen umfassen. Einzelne Zusatzstoffe von besonderer Bedeutung (Menthol, Glyzerin, 1,2-Propylenglykol, Sorbit, Zucker, Kakao, Lakritze, Zitronensäure, Triazetin und Ammoniumverbindungen) werden in eigenen Kapiteln besprochen, die durchgängig in spezielle Abschnitte unterteilt sind: Verwendung und toxikologische Bewertung; Einsatzmenge in Zigaretten, Transfer und Pyrolyse; Attraktivität und Suchtpotential; Auswirkungen auf die Zusammensetzung des Hauptstromrauches von Zigaretten; Auswirkungen auf die Toxizität des Hauptstromrauches von Zigaretten. Epidemiologische Befunde und Daten aus Biomonitoring-Studien mit Rauchern von Zigaretten mit und ohne Zusatzstoffe werden referiert und diskutiert, insbesondere für American blend-Zigaretten, Virginia-Zigaretten, "Französische" (schwarze) Zigaretten und Menthol-Zigaretten, wobei die Auswirkungen von Zusatzstoffen auf das Rauchverhalten und mögliche Gesundheitsrisiken im Mittelpunkt stehen.

In jüngerer Zeit wurden zweckbestimmte Übersichten veröffentlicht, deren Wert durch die willkürliche Auswahl von Quellen und durch einseitige Sichtweise beeinträchtigt ist. Läßt man diese Übersichten außer Acht, zeigt der zusammengefasste wissenschaftliche Kenntnisstand, dass Zusatzstoffe im Tabak nur gelegentliche und begrenzte Auswirkungen auf die Zusammensetzung des Hauptstromrauches von Zigaretten haben, die sich fast niemals auf die Ergebnisse toxikologischer *in vitro*-Prüfungen oder *in vivo*-Studien auswirken. Dies bekräftigt die Schlussfolgerung, dass von Zusatzstoffen im Tabak nicht anzunehmen ist, sie würden die bekannten Gesundheitsrisiken des Rauchens verstärken. Ebenso fehlt ein Beweis für die Behauptungen, dass bestimmte Zusatzstoffe die Verfügbarkeit oder das Suchtpotential von Nikotin erhöhten. [Beitr. Tabakforsch. Int. 25 (2012) 411–493]

RESUME

Les additifs dans le tabac jouent un rôle prépondérant dans la fabrication et pour la qualité des produits du tabac, en particulier des cigarettes et des tabacs à rouler. Une attention de plus en plus grande est accordée aux effets possibles des additifs sur le comportement et la santé des consommateurs. Le présent aperçu a pour objectif de rassembler, de classifier et, dans une certaine mesure, d'évaluer les innombrables informations scientifiques relatives à ce sujet qui sont disponibles dans les publications et les autres sources spécialisées. D´abord, les raisons de l'utilisation d'additifs dans la fabrication de cigarettes sont exposées. En réponse à la controverse grandissante concernant l'attractivité et le potentiel addictif du tabac, le compte rendu propose une clarification de termes et de concepts, qui est complétée par la discussion détaillée de deux groupes de substances particulièrement pertinents: les additifs tels que les composés d'ammonium, dont il est prétendu qu'ils augmentent la disponibilité de la nicotine, et les additifs qui sont prétendus renforcer le potentiel de dépendance à la nicotine.
La composition et la toxicité de la fumée principale des cigarettes avec et sans additifs sont examinées sous plusieurs aspects. L'observation de toute une série d'études clés permet d´élucider la valeur des études de pyrolyse, suivi d´une discussion sur certaines réflexions fondamentales relatives aux contrôles de toxicité in vitro et in vivo. Cinq revues majeures de la littérature relative aux additifs, qui ont été publiées entre 1994 et 2004, ainsi que les résultats de plusieurs études expérimentales, publiées entre 2002 et 2012 et couvrant une grande partie des additifs, font l'objet d'une discussion détaillée. Certains additifs particulièrement importants (menthol, glycérol, 1,2-propylène glycol, sorbitol, sucres, cacao, réglisse, acide citrique, triacétine et composés d'ammonium) sont traités dans des chapitres spécifiques, qui sont systématiquement subdivisés en sections spéciales: utilisation et évaluation toxicologique; quantité utilisée dans les cigarettes, transfert et pyrolyse ; attractivité et potentiel de dépendance ; effets sur la composition de la fumée principale des cigarettes ; effets sur la toxicité de la fumée principale des cigarettes. Les résultats épidémiologiques et les données d'études de biosurveillance menées sur des fumeurs de cigarettes avec et sans additifs ont été rassemblés et examinés spécialement pour les cigarettes américaines (“American blend”), les cigarettes Virginia, les cigarettes “françaises” (noires) et les cigarettes au menthol, l'accent étant mis essentiellement sur les effets des additifs sur le comportement tabagique et les risques éventuels pour la santé.
Ces dernières années ont vu la publication d'exposés partiaux dont la valeur est compromise par la sélection arbitraire des sources et par une évaluation déséquilibrée. Si l'on fait abstraction de ceux-ci, l'état des connaissances scientifiques montre en résumé que les additifs dans le tabac ont uniquement des effets occasionnels et limités sur la composition de la fumée principale des cigarettes, qui ne se manifestent presque jamais dans les résultats des essais toxicologiques *in vitro* ou les études *in vivo*. Ceci renforce la conclusion selon laquelle on ne peut supposer que les additifs dans le tabac renforcent les risques connus du tabagisme pour la santé. De même, il n'existe aucune preuve concernant les affirmations selon lesquelles certains additifs augmenteraient la disponibilité ou le potentiel addictif de la nicotine. [Beitr. Tabakforsch. Int. 25 (2012) 411–493]

1. INTRODUCTION

While there is a considerable degree of inconsistency in the scientific and “political” literature, the use of the terms “additive” and “ingredient” in this review follows strictly the rules of logics and semantic definitions. An additive is “*a substance added to something in small quantities, typically to improve or preserve it*” and an ingredient is “*a component part or element of something*” (1). It follows that tobacco additives are all materials added to tobacco in the various processing and manufacturing steps whereas tobacco ingredients are the components, which are naturally inherent in tobacco (it remains debatable whether agrochemical residues are covered by this definition). Logically, tobacco additives become tobacco product (cigarette) ingredients upon completion of the manufacturing process. Actually, it makes little sense to use the term “cigarette additive”. In essence, this line of thoughts is in compliance with the following definitions:

- According to the EU Directive 2001/37/EC (2) ingredients in tobacco products are defined as all materials added to tobacco and used for tobacco products, including filters, papers, glues, inks and aroma substances.
- In the draft joint guidelines for the implementation of Articles 9 and 10 of the WHO FRAMEWORK CONVENTION ON TOBACCO CONTROL (FCTC) (3), the definition is expanded and now includes “*tobacco, components (e.g. paper, filter), including materials used to manufacture those components, additives, processing aids, residual substances found in tobacco (following storage and processing), and substances that migrate from the packaging material into the product (contaminants are not part of the ingredients).*”

This review is focused on materials directly added to tobacco and used in the process of manufacturing finished products, starting with the blending of different raw tobaccos. Residues of agrochemicals and pesticides resulting from tobacco cultivation and pest control during storage, processing aids and contaminations from packaging materials do not serve the purpose of modifying or preserving product properties and are, therefore, not considered.
There is a long tradition of adding aromatic substances in tobacco product manufacturing. Already in the 17th century, essential oils, such as orange oil and bergamot oil, were used for nasal snuff production in Saxony and Poland (4). In the 18th, 19th and 20th centuries, tobacco companies added additives also to pipe and chewing tobaccos for imparting a specific taste, flavor or aroma on the product (5, 6) or for technological reasons, such as increasing the moisture holding capacity of the tobacco (7). The first reports suggesting that tobacco additives, such as glycerol, sugar and “oils”, may have an influence on the composition of tobacco smoke - and specifically increase aldehyde levels - were published as early as 1912 in the British

medical journal THE LANCET (8). It was speculated that certain additives could have an effect on the health of smokers.

In cigarette manufacturing tobacco additives were first used in the U.S.A. at the beginning of the twentieth century. The first cigarette manufactured from a cased and flavored blend of flue cured Virginia, air cured Burley and sun cured Oriental tobaccos, the 70 mm Camel brand, was introduced by the R.J. Reynolds Tobacco Co. in 1913 (9). Today, this type of cigarette, called American blend cigarette, has the highest market share in Europe and worldwide. In nearly all countries with the exception of China, which were not associated with the British Empire, mostly American blend cigarettes are being consumed (10). In the countries, which are or were part of the British Commonwealth, e.g., Great Britain, Australia, South Africa and Canada, flavor additives are not generally used on cigarettes (11). Nearly all tobacco blends of these cigarettes consist of flue cured Virginia tobaccos without any additives.

Since the 1960s, there is an enduring discussion in the scientific literature whether additives used for cigarette manufacturing elevate the health risks of smoking (12). In 1967, WYNDER and HOFFMANN (13) hypothesized that tobacco additives in cigarettes may increase or decrease the toxicity of the smoke. The allegation of increased risk was repeated several times (14, 15). Based on this vague hypothesis consequences were called for (16, 17). In the draft joint guidelines for the implementation of Articles 9 and 10 (Regulation of the contents of tobacco products and regulation of tobacco product disclosure) of the WHO FRAMEWORK CONVENTION ON TOBACCO CONTROL (FCTC) the prohibition, or at least the strong restriction, of the use of aromatic additives in cigarette manufacturing was proposed (3). The prohibition of specific aromatic additives by law was recently implemented in Canada (18). Besides reducing smoking related health risks for cigarette consumers it was speculated that the prohibition of specific additives would make cigarettes less attractive and less addictive, especially protecting children and young people from starting to smoke (17).

In this review, published data on the influence of tobacco additives on smoking related health risks are compiled and discussed. The overview is focused on substances added to tobacco in the manufacturing of cigarettes. To a large extent it is relevant also for the tobacco additives in roll-your-own products.

2. REASONS FOR THE USE OF TOBACCO ADDITIVES IN CIGARETTE MANUFACTURING

There are specific reasons for using additives in cigarette manufacturing. Additives for particular technological purposes, such as increasing the moisture holding capacity of the filling tobacco, are used in all manufacturing processes of traditional American blend cigarettes and sometimes also for Virginia and the "French" cigarettes made from dark air cured tobaccos. In the past, the use of additives for imparting a specific taste, flavor or aroma on the product was common practice for all American blend cigarettes. Recently, however, certain American blend (U.S. style) cigarette brands without any tobacco additives, even with no humectants, were introduced into the market. In general, no flavoring additives are used in making the tobacco blends of Virginia and dark "French" cigarettes.

Figure 1 shows schematically a typical production facility for American blend cigarettes. It consists of three different lines, the leaf line for blending and processing the Virginia, Oriental and reconstituted tobaccos, the Burley line and the stem line for rolling, cutting and expanding the mid-ribs of Virginia and Burley tobaccos. The processed tobacco strips of the leaf and Burley lines are blended and cut, followed by the final blending with the expanded cut rolled stems and expanded tobacco.

There are several techniques for manufacturing reconstituted tobaccos for use in the leaf line. Stems and fines of tobacco are combined with cellulose fibers or cellulose derivatives as binders to form sheets, which can then be treated and processed like natural tobacco. This means that specific additives are in use for manufacturing reconstituted tobaccos (19, 20).

During processing additives may be added to the tobacco at different points. In Figure 1, these are marked in grey. The casing added to the tobacco blend of the leaf line (with different Virginia, Oriental and reconstituted tobaccos) consists primarily of humectants, such as glycerol, 1,2-propylene glycol or sorbitol, fruit extracts, sugars, cocoa, licorice, etc. These substances are water soluble or can be suspended in water. They strengthen the "basic" tobacco taste and provide the brand's characteristic smoke aroma without changing the chemical composition of the raw tobaccos used for the blend.

The addition of humectants, their kind and levels depend on cigarette type and especially on the quality and structure of the tobaccos. Humectants make tobacco softer and reduce the rigidity and, consequently, the brittleness of tobacco leaves and cut tobacco during cigarette manufacturing. The main purpose, however, is maintaining the moisture content of tobacco in the finished cigarette - usually around 12% at the time of manufacturing. If cigarettes lose moisture and become "dry" during transport and storage, slight changes in smoke composition may result and the taste of the smoke may become harsh, "strong" and more irritating (21).

Generally, glycerol, 1,2-propylene glycol, triethylene glycol and sorbitol or mixtures of these substances are used as humectants. Most common is the use of a mixture of glycerol and 1,2-propylene glycol at a level of up to 4.5% on tobacco (21).

Unlike other humectants, glycerol is a naturally occurring tobacco component (0.07–0.48% depending on tobacco type) (21). Pure glycerol has a sweet taste. However, at the levels used in cigarettes glycerol lacks the potency of shifting the taste of cigarette smoke in a "sweet" direction. Sorbitol is a component of the rowan berries and other berries, such as raspberries (22, 23). The other humectants, 1,2-propylene glycol or triethylene glycol, are synthetic compounds and not found in nature.

The tobaccos of the second line, the Burley line, are treated with a casing sauce, which also consists of water soluble substances or substances suspended in water. Sugars, fruit acids, such as citric and tartaric acid, cocoa and licorice are the main components of the sauce; occasionally, other compounds are used in rather small amounts. Air cured Burley tobaccos are characteristically low in reducing

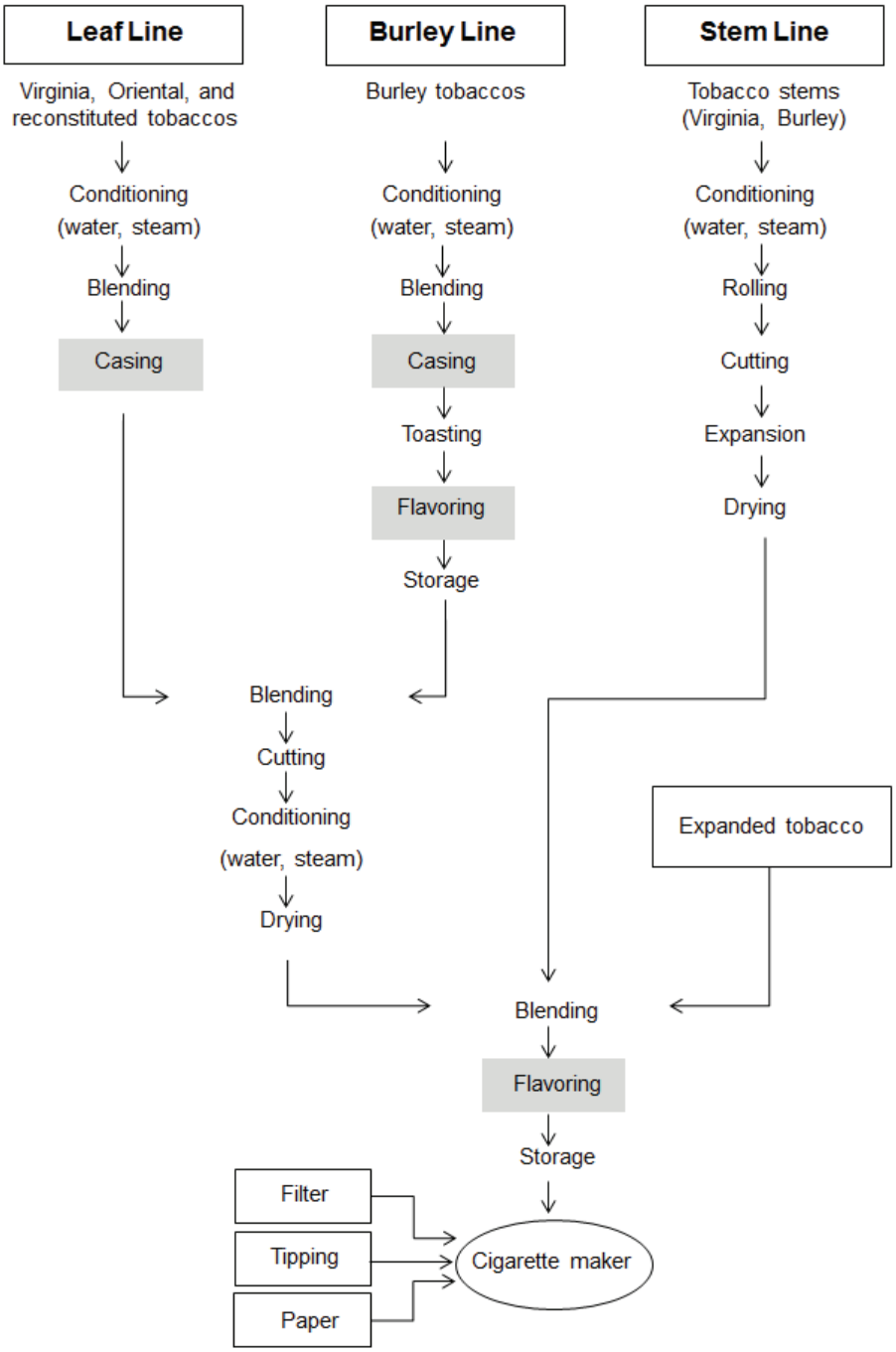

Figure 1. Typical production scheme for American blend cigarettes.

sugars (0.2% or less) (24). Most types of Burley tobacco used in American blend cigarettes produce strong, pungent smoke. The addition of sugars together with other casing components improves the overall smoking quality of these tobaccos (25–29).

A special piece of equipment, the toaster, forms the center of the Burley line. Here, the cased Burley tobacco is dried from more than 50% to about 4% humidity in a heated drum with a wall temperature of around 160 °C. After passing a cooling zone the tobacco is re-moistened to around 25% humidity. Subsequently, additional aromatic additives ("Burley flavor") can be added to the toasted tobacco.

Resulting from the air curing process Burley tobaccos may be rich in unpleasant degradation products, such as ammonia and aliphatic amines, which are partly generated from proteins. Burley tobaccos are also rich in nitrate (24). During curing nitrate is microbially reduced to nitrite and, as a consequence, nitrosamines are formed by reactions with secondary and tertiary amines (aliphatic amines, alicyclic amines or tobacco alkaloids) (30–33).

In contrast to the tobacco treatment in the leaf line (application of casing only), the chemical composition of the tobacco itself is changed by Burley toasting, with a direct influence on smoke. Ammonia and volatile nitrogen bases generated during the curing process (34) are partly removed by heat treatment during toasting resulting in lower levels of these components in smoke (25). Traces of volatile *N*-nitrosamines, such as *N*-nitrosodimethylamine, *N*-nitrosomethylethylamine and *N*-nitrosopyrrolidine, may also be detected in untreated Burley tobacco (35). These nitrosamines are steam volatile; a distinct reduction is observed in Burley tobaccos after toasting (36).

Besides reducing ammonia, nitrogen bases and certain other volatile compounds in the tobacco, the most important step in improving the flavor and smoking quality of Burley tobacco by toasting is the reaction between reducing sugars and ammonia, amines and amino acids. BRIGHT *et al.* (37) demonstrated that the weight percentages of five amino acids (aspartic acid, proline, lysine, histidine, arginine) in tobacco were reduced by heat treatment, while the content of dimethyl pyrazines increased dramatically, e.g., from 0.1 ppm to 460 ppm.

The function of amino acids and sugars in the production of volatile compounds, such as carbonyls and pyrazines, by the heat treatment of tobacco was studied in model experiments by COLEMAN and PERFETTI (38). Their role was seen in two major reaction pathways: the Strecker degradation of amino acids (39) and Maillard type reactions between amino acids and sugars (40, 41).

Recently, LI *et al.* (42) reported the decrease of free amino acids by 16.5–20.7% in Burley tobaccos when toasted. Parallel to the decrease of amino acids due to their consumption in the Maillard reaction (43), the amount of pyrazines was increased during toasting. The substances mentioned above, sugars, ammonia, pyrazines and free amino acids - all affected by the toasting process, are natural tobacco constituents. Following treatment in the Burley toaster the quantitative ratios of these compounds are changed.

Licorice extracts are produced by extracting licorice roots with water. Besides sugars, the extracts contain glycyrrhizin, a mixture of the potassium and calcium salts of glycyrrhizic acid, considered to be the primary flavor constituents (44, 45) together with several flavorful alkylpyrazines (21). After filtration, the licorice extract is either concentrated to a syrupy consistence, spray-dried forming a powder or made into a solid block after the complete evaporation of water (46).

Licorice has been used since the 1880s as an additive in tobacco product manufacturing (47). Usually, up to 1% of licorice is added to tobacco (21), enhancing and harmonizing smoke flavor, reducing the dryness in the smoker's mouth and throat and improving the moisture holding characteristics of tobacco. The rough character of tobacco smoke is minimized by balancing its overall flavor profile. In addition, licorice acts as a surface active agent during the application of casing sauces, in both the leaf and Burley lines, uniformly improving the rate of adsorption of additives by the tobacco (46).

Cocoa powder is widely used as an important component in casing solutions for cigarette manufacturing, improving the taste and aroma of smoke. Usually about 1% cocoa is applied to tobacco (48). Cocoa, like licorice, contains numerous pyrazines as well as theobromine and traces of caffeine (21). Cocoa and its pyrolysis and combustion products contribute to the characteristic flavor and taste of American blend cigarettes.

Besides the application of additives, tobacco processing in cigarette manufacturing consists of sequential conditioning with steam and water, and heat treatment. The objective is to improve and maintain the elasticity of the cut tobacco fibers and, consequently, their filling power. Filling power is directly related to the firmness of a cigarette, an important quality parameter.

The processing of the mid-ribs of Burley or Virginia tobaccos has the same goal. The stems are broken into small pieces (3–5 cm in length), conditioned with water and steam, rolled, cut and expanded by flash drying. The water, evaporating from the cells, expands the rolled cut stems to double their volume. The impregnation of cut stems before drying with an aqueous ammonium hydrogen carbonate solution as an expansion aid (like baking powder) is described in certain patents (49). Before flavoring, the cut and expanded stems and expanded tobacco are combined with the cut tobaccos produced in the leaf and Burley lines.

Several techniques are available for producing expanded tobacco. In principle, cut tobacco is soaked with a low boiling liquid and flash dried afterwards. The evaporating liquid expands the cell structure of the tobacco fibers up to two-fold the original volume. Today, liquid propane and pentane (50) and liquid carbon dioxide (51) are commonly used as "processing aids". Another process for expanding tobacco uses nitrogen and/or argon. Treatment of tobacco is done in an autoclave at pressures up to 1000 bar, with subsequent decompression (52). The first expansion process for cut tobacco was the G-13 process developed by the R.J. Reynolds Tobacco Co. (53), which used Freon 11® (trichlorofluoromethane) as expansion agent. In 1975, ROWLAND and MOLINA (54) reported that Freon 11® and other chlorofluoromethanes were involved in thinning out the ozone layer. This discovery ultimately resulted in the banning of most commercial uses of low molecular weight chlorofluoromethanes, including their use in the G-13 process.

One of the first investigations of the effect of expanded tobacco on cigarette mainstream smoke toxicity was done in 1977 by DONTENWILL *et al.* (55). The tumorigenic activity of smoke condensate from cigarettes containing 20% expanded tobacco was evaluated on CFLP mouse skin. Compared to the smoke condensate generated from reference cigarettes without expanded tobacco, significantly lower tumorigenic effects were observed.

The toxicological effects of expanded tobacco as used in today's cigarettes were evaluated by COGGINS *et al.* (56) and by THEOPHILUS *et al.* (57–59). Testing included mainstream smoke chemistry, genotoxicity, a 13-week inhalation study in Sprague-Dawley rats and a 30-week dermal tumor promotion study in SENCAR mice. The data demonstrated similar toxicological profiles for the mainstream smoke of cigarettes with and without expanded tobacco.

Prior to the final manufacturing step, top dressing (top flavor) is usually sprayed on the processed and cut tobacco

blend, generally in a drum and as an alcoholic solution at a level of 0.5–1.5% of tobacco weight. It consists of a complex mixture of several aromatic substances, mostly at ppm levels, giving a cigarette brand its unique sensory characteristics.

In more concentrated form the top dressing can be applied directly onto the tobacco rod of the cigarette. This technique uses a fine jet on the cigarette maker spraying the top dressing solution directly onto the tobacco rod (60). Another way of cigarette flavoring uses a cotton thread loaded with aroma substances placed in the center of the cigarette filter plug. This approach is primarily used for manufacturing mentholated cigarettes (61). Menthol dissolved in the acetate filter additive, triacetin, and applied during filter manufacturing is another technique for producing mentholated cigarettes. These can also be made by impregnating the paper (laminated on aluminum foil) of the cigarette pack with menthol (62). The menthol migrates quickly from the package into the filter and the tobacco rod of the cigarettes (63). A review of the technologies related to menthol use in cigarette manufacturing was prepared by BORSCHKE (64).

Additives are also present in cigarette filters and papers. Most modern cigarette filters consist of cellulose acetate fibers of various specifications. To bind the fibers together, 7–8% of the esters, triacetin (glycerol triacetate) or TEGDA (triethyleneglycol diacetate), is added. Triacetin and TEGDA are also important for modifying the composition of cigarette mainstream smoke. The presence of these esters in the cellulose acetate filter is responsible for the selective reduction of simple phenols, pyridines, quinolines and - last but not least - volatile *N*-nitrosamines, such as *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine, in tobacco smoke (65).

As a residue from fiber production traces of the technical aid, spinning oil (highly purified paraffin oil), may be detected in filters. About 1.5% of titanium dioxide (anatase) is incorporated into the cellulose acetate fibers. It has no active function in cigarette smoke filtration; the titanium dioxide pigments are primarily used as delustering and whitening agent in the cellulose acetate fibers. As a photo-catalyst the pigments are also responsible for the photochemical degradation of the filter fibers (66).

Activated carbon is another filter component influencing the composition of cigarette mainstream smoke. It shows significant adsorption activity for many gas phase constituents present in mainstream smoke (67, 68). The yields of carbonyl compounds in mainstream smoke, such as formaldehyde, acetaldehyde and acrolein, were reported to be reduced by 80, 90 and 95%, respectively. Other toxicants of the mainstream smoke gaseous phase, such as hydrogen cyanide, 1,3-butadiene, isoprene, benzene, were found to be reduced between 90 and 95% (69). Filter efficiency is dependent on the amount and quality of the activated carbon used (70). It has been demonstrated that the retention capacity of the carbon may decrease during the first 2 weeks during storage of the manufactured cigarettes (71). As shown recently by PURKIS *et al.* (72) the smoking regimen also has an influence on the retention capacity of carbon filters. Compared to the ISO machine smoking regimen (73), the Canadian Intense regimen (74) with 100% tip ventilation blocking made the adsorption of vapor phase components of cigarette mainstream smoke by carbon filters less efficient. The explanation for the unequal retention capacities may be the different flow velocities of the smoke through the carbon part of the filter: 35 mL in 2 sec without vent blocking (ISO) or 55 mL in 2 sec with 100% vent blocking (Canadian). Consequently, the contact time between the gas phase of the smoke and the activated carbon is shorter with the Canadian regimen reducing retention efficiency. In addition, PURKIS *et al.* (72) observed an increase in mainstream smoke temperature in the last puffs when cigarettes were smoked under the vigorous Canadian Intense regimen (74). This resulted in reduced vapor phase adsorption on the carbon and even some desorption during later puffs. Saturation of the active carbon adsorption sites in fhe filter was not considered to be of importance.

PAULY *et al.* (75–78) speculated that charcoal particles and parts of cellulose acetate fibers released from cigarette filters could be aspired during smoking and retained in parts of the respiratory tract, in particular the lungs. In response to these assertions, studies were performed by the industry concerned. An overview of studies concerning fiber release was prepared by HENGSTBERGER and STARK (79). The findings revealed that some acetate fiber fragments were generated during the manufacturing of the cigarettes. During smoking they remain primarily in the filter. Any fibers potentially released during puffing would be deposited inside the oral cavity because they are too large for passing the larynx and entering the bronchial or pulmonary sections of the respiratory tract. Less than 10 fiber particles per cigarette (diameter > 0.3 µm and length not exceeding 12 µm) are usually released during smoking. A working group of the GERMAN STANDARDIZATION ORGANIZATION (DIN) concluded after evaluating the relevant experimental data with special attention to fiber-shaped particles (80): "*From the toxicological perspective, compared to the health risks otherwise associated with cigarette smoking, the release of particles from acetate filters does not constitute a particular health risk*".

AGYEI-AYE *et al.* (81) investigated the contribution of the charcoal fall-out from cigarettes equipped with activated carbon filters to the overall amount of particles and fibers released from filters during smoking. They showed that compared to the numbers quoted by others (76, 82, 83) only a small amount of charcoal was released. During smoking these particles had a low likelihood of reaching the lungs.

Regarding the use of additives in cigarette papers, burning velocity and porosity are controlled by calcium carbonate in combination with alkali citrates, acetates or ammonium phosphates. About 20% calcium carbonate and around 0.5–2% alkali citrates or acetates are commonly incorporated in the paper. Cigarettes must be self-extinguishing when distributed in the member states of the European Union since 2011, with no precise date set for implementation (84), or in other parts of the world, for instance in a number of States of the U.S., like New York State since 2004 (85). The requirement is met by using a specific cigarette paper (low ignition propensity = LIP paper), which may contain specific additives. The most common way towards this feature is including narrow zones of extremely low air permeability in the paper, which results

in self-extinguishing of the cigarette when the glowing cone reaches such zones between puffing (banded cigarette paper technology). The toxicity of the mainstream smoke of cigarettes equipped with LIP paper was compared to cigarettes with common cigarette paper. THEOPHILUS *et al.* (86) used the established toxicity testing program of R.J. Reynolds Tobacco Co. (mainstream smoke chemistry, *in vitro* genotoxicity and cytotoxicity, 13-week nose-only inhalation in Sprague-Dawley rats, 30-week dermal tumor promotion in SENCAR mice). No difference in cigarette mainstream smoke toxicity was seen comparing cigarettes equipped with the two kinds of paper. Comparable results were reported by APPLETON *et al.* (87), LULHAM *et al.* (88), MISRA *et al.* (89), and PATSKAN *et al.* (90).
Adhesives are required for gluing the cigarette paper seam and attaching the filter to the tobacco rod. Polyvinyl acetate and starch are materials suitable for the purpose.

3. TOBACCO ADDITIVES AND THE ADDICTIVENESS AND ATTRACTIVENESS OF SMOKING

There are efforts on the regulatory side to understand and evaluate whether additives contribute to or increase the addictive properties of tobacco products. This is an objective of the WHO Framework Convention on Tobacco Control (FCTC), which calls for regulations of the contents and emissions of tobacco products and addresses the reduction of their attractiveness and addictiveness (3). Guidelines for the regulation of the contents of tobacco products including the additives used for manufacturing were put forward.
In the Tobacco Products Directive of the EUROPEAN UNION 2001/37/EC (2) it is 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. Since 2001, the Directive has challenged the Commission to propose a common list of additives authorized for tobacco products, taking into account, *inter alia*, their addictiveness. In its Second Report on the implementation of the Tobacco Products Directive the Commission stresses the need for further work on the addictiveness of tobacco additives (91).
In 2009, the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) was asked by the Commission of the European Union to evaluate the role of tobacco additives in the addictiveness and attractiveness of tobacco products. The report of SCENIHR was published in November 2010 (92).
This review does not intend to discuss the commonly used definitions for tobacco dependence and the assumptions of the addictiveness of tobacco or nicotine. Only the contribution of additives to the addictiveness and attractiveness of tobacco products and some of the difficulties and general conclusions of the experts of SCENIHR are mentioned and discussed in the following.

3.1. Addictiveness

In 1964, a WHO Expert Committee introduced the term "dependence" to replace the prevailing terms "addiction" and "habituation" (93). Nevertheless, the term addiction is still commonly used when referring to what is technically known as dependence. Addictiveness refers to the pharmacological potential of a substance to cause addiction (94). The terms "dependence causing" and "dependence potential" are often used as synonyms for "addictive" and "addictiveness".
Dependence was defined by the WHO EXPERT COMMITTEE ON DRUG DEPENDENCE (94) and the ICD-10 (Tenth Revision of the International Classification of Diseases and Health Problems) Classification of Mental and Behavioural Disorders: Clinical Descriptions and Diagnostic Guidelines (95).
It is currently believed that "tobacco addiction" is maintained by the pharmacological substance, nicotine. According to SCENIHR (92, on page 64) "*tobacco products that do not deliver nicotine do not sustain addiction*".
Animal models have been widely used to investigate the neurobiological basis of nicotine addictiveness and the results obtained suggest that the neurobiological activity of nicotine is complex involving various neurotransmitter pathways. Nicotine was found to have relatively low addictive potency in animal studies and it was concluded that the risk of addiction to pure nicotine in humans would also be low, contrary to the high addictive potential of tobacco products. However, there are no experimental data in humans regarding nicotine addiction (92).
No animal models are available for investigating and assessing the various aspects of human tobacco dependence (96, 97); human studies are the only feasible approach (98). Most studies used either the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria (99) for assessing human tobacco dependence or a proxy measure such as the FAGERSTROM Test for Nicotine Dependence (100), or the time to first cigarette in the morning or the number of cigarettes smoked per day (101). In the view of many scientists the studies confirm that tobacco - and not nicotine - has high addictive potential in humans.
For explaining the difference between the addictive effects of nicotine and tobacco products in humans many scientists believe that there is something in tobacco or tobacco smoke that increases the addictiveness of nicotine but less attention is paid to the possibility of external factors of tobacco use contributing to addictiveness. Instead, there is a focus on the question whether additives increase the addictive potential of nicotine in the finished tobacco product. In theory, the addictive potency of tobacco products may be directly increased by additives provided certain additives have intrinsic addictive properties. Other possibilities are indirect factors like additives that facilitate the inhalation of tobacco smoke, additives that enhance the nicotine content of smoke, additives that increase the bioavailability of nicotine, or additives that generate compounds in mainstream smoke, which increase the addictiveness of nicotine.
In 2010, after more than one year of evaluating the relevant literature, the SCENIHR Working Group published their conclusions regarding the addictiveness of tobacco additives. In their final opinion the Working Group pointed out: "*The criteria for dependence established in humans indicate that tobacco has a high addictive potential, but it remains difficult to assess the addictiveness of individual additives. In animal studies the addictive potency of the finished tobacco product cannot be assessed. ... In humans, the positive correlation between tobacco consumption and dependence suggests that individuals with high nicotine levels in their blood are more dependent.*" (92, on page 4).

"*At present it is not possible to evaluate whether additives increase the addictive potency of the final tobacco product. ... No tobacco additives which are addictive by themselves have so far been identified. ... In conclusion, apart from the possible action of combustion products of sugars (acetaldehyde and similar compounds that enhance the action of nicotine by inhibition of MAO* [monoamino oxidase] *), there is no evidence as yet that additives enhance the addictiveness of nicotine and therefore of tobacco. ... In conclusion, the methods used to quantify the potency of additives in humans or animals have limitations, and the available methodologies are thus not considered adequate for a reliable quantification.*" (92, on page 82-84).

In our opinion, the addictive potency of nicotine is not a sufficient explanation for the dependence of smokers on tobacco products. Additional pharmacological and behavioral effects of tobacco consumption have to be factored in. They interact strongly and are the basis for the success of tobacco products on the market.

The working hypothesis of the unique role of "nicotine uptake" in the addictiveness of tobacco smoking represents a very reduced and simplified perspective. Other aspects of using tobacco products or tobacco smoking may also play a role in the habituation of becoming a smoker. However, despite the conclusions of the SCENIHR Working Group (92), certain additives like ammonia, sugars and their combustion products and menthol continue to be discussed as enhancers of nicotine and tobacco product addictiveness. These questions and the available pertinent data are discussed in the following.

3.2. Tobacco additives like ammonium compounds that are alleged to increase nicotine availability

The route of nicotine uptake, its pharmacokinetics and bioavailability are in part responsible for the stimulating effects of smoking observed in humans. The faster the release of nicotine from the tobacco product, the rate of absorption and the attainment of peak levels, the higher is the likelihood of continued use or abuse by humans (102). When smoking cigarettes, nicotine is taken up rapidly by the lungs and to a certain degree also by the oral mucosa. With the exception of some cigar types and cigarettes made from dark air cured tobaccos, cigarette mainstream smoke shows a slightly acidic reaction (103). Under these pH conditions the nicotine molecule is predominantly protonated and found in the particulate phase of smoke (104). Unprotonated nicotine, some of which may be present in minute amounts in the vapor phase of smoke depending on cigarette construction and tobacco blend, is absorbed rather quickly through the mucosal membranes of the oral surface and the lungs (105).

Reducing the acidity of cigarette mainstream smoke for a more alkaline reaction is expected to convert di-protonated nicotine to mono-protonated and unprotonated, free-base nicotine and may, therefore, enhance its effects. This was heavily discussed internally by scientists of the tobacco industry (106, 107). It was speculated that low nicotine cigarettes with more alkaline smoke might produce more free nicotine and, consequently, have higher nicotine impact (108). Ammonia and ammonia releasing compounds were thought to be agents for increasing smoke pH (109, 110).

The unproven hypotheses and concepts of a number of tobacco industry scientists, recorded in unpublished internal company documents (106–110), concerning "ammonia as a tobacco additive", supposedly increasing the nicotine impact in smokers, were adopted enthusiastically by tobacco control organizations. They presented them as "facts" and considered them as evidence that the cigarette industry manipulated cigarettes to increase their addictiveness (15, 17, 111–113). Advocates are sticking to this line of accusations until today (3, 114–116).

Fortunately, there are a number of solid scientific studies, which shed light on the challenging problems of nicotine chemistry and physiology.

In 1997, RICKERT (117) characterized 10 different leading U.S. cigarette brands. Ammonium ions were present in the tobacco of these American blend cigarettes at levels of 0.11–0.34%. In evaluating the ammonium content of tobacco, it must be taken into account that ammonia and ammonia releasing components are natural constituents of tobacco (24). In mainstream smoke, ammonia levels of 1.36–34.15 µg/cig were measured by RICKERT. The wide range is due to cigarette construction and blend. There was a correlation between mainstream smoke condensate and mainstream smoke ammonia values. Interestingly, the measurements of the so-called "smoke pH" showed a remarkably narrow range between 6.025 and 6.325. At this pH range the ratio between protonated and un-protonated nicotine in smoke is about 99 to 1. The effect of filter ventilation on mainstream "smoke pH" was shown in 1981 by KLUS *et al.* (118). Increasing the degree of filter ventilation led to higher mainstream smoke pH values.

In 1999, ELLIS *et al.* (119) investigated the effect of ammonia compounds added to cigarettes on "smoke pH" and on the mainstream smoke levels of nicotine and ammonia. In a series of experimental cigarettes with increasing amounts of ammonia in the tobacco blend, a significant increase of ammonia in smoke was observed. However, these increases were not as apparent in "smoke pH" and showed no correlation with the amount of nicotine in smoke. Therefore, the ammonia content of cigarette mainstream smoke is not a significant factor in mainstream "smoke pH". In 2006, the findings of ELLIS *et al.* were confirmed and expanded by CALLICUTT *et al.* (120). They concluded that neither "tobacco pH" nor "smoke pH" had scientific or practical relevance for the transfer of nicotine from tobacco to smoke in the cigarettes used in their study. The complex knowledge about "smoke pH", its determination and influence on the biological activity of mainstream smoke and on certain aspects of mainstream smoke chemistry, and the effects of the ammoniation of tobacco on cigarette mainstream smoke properties were reviewed by RODGMAN in 2000 (121).

The role of gas/particulate partitioning in the deposition of nicotine and other tobacco smoke compounds in the respiratory tract was discussed in depth by PANKOW (122). Four different mechanisms had to be considered: First, the direct deposition of nicotine initially present in the gas phase of inhaled smoke; second, evaporation of nicotine from the particulate phase followed by deposition; third, deposition of smoke particles and evaporation of nicotine

from the deposited particles followed by nicotine deposition; and fourth, particle deposition with nicotine diffusion into the respiratory tract tissue. In view of the volatility of nicotine, the first three mechanisms were judged to be particularly important - the fourth (particle deposition followed by diffusion) being most relevant for non-volatile compounds such as PAHs. The paper included a discussion of the role of the "effective pH of particulate phase" in nicotine absorption.

For evaluating the mechanisms of nicotine uptake during smoking, the partitioning of nicotine and ammonia between the particulate and gas phases of cigarette mainstream smoke was studied by INGEBRETHSEN *et al.* (123). Particulate-gas equilibria were determined by diffusion denuder collection. The surface deposition rate of nicotine was shown to decrease as the smoke traversed the denuder. This effect was attributed to the changing vapor pressure of the nicotine in the particles, driven by the rapid loss of volatile ammonia from the particles. Dilution of mainstream smoke enhanced the fractional deposition of both nicotine and ammonia in the denuder tubes.

While the analytical data obtained with experimental and commercial cigarettes obviously did not confirm the "ammonia hypothesis" discussed by tobacco industry scientists, neither the industry nor tobacco control organizations had paid much attention to the physiological situation in the human lungs. It is indeed known that nicotine is rapidly absorbed in the lungs, independent from "smoke pH" (124–128). This is to say, irrespective of whether (and to which degree) nicotine in the smoke is protonated or not. This was further explored in 2000 by DIXON *et al.* (129). They pointed out that the considerable buffering capacity (7 mval/pH unit) of the lung surface liquid at pH 7.4 (130) is not exceeded when nicotine reaches the lungs in amounts between 0.01 and 0.1 mg per puff. Therefore, after hitting the lung surface nicotine always exists in a 20:80 ratio of un-protonated:mono-protonated nicotine regardless of the form it enters the lungs.

A small study by ARMITAGE *et al.* (131) showed in 2004 that the treatment of cigarette tobacco with urea or diammonium hydrogen phosphate failed to increase the venous blood levels of nicotine in smokers compared to additive free control cigarettes. The study investigated the delivery and respiratory tract retention of nicotine and solanesol from cigarettes containing urea or diammonium hydrogen phosphate in ten smokers under the conditions of mouth-holding and inhaling volumes of 75 mL and 500 mL. Nicotine retention in the mouth during mouth-holding was significantly higher for ammoniated cigarettes (64.3 ±10.5%) than for those with urea (53.3 ±11.3%) or control cigarettes (46.3 ± 8.6%). This, however, did not result in increased nicotine levels in venous blood.

In 2006, WILLEMS *et al.* (132) examined the wealth of available literature (both published papers and released industry documents) in a review focused "*on the hypothesis that the addition of ammonium compounds to tobacco enhances global tobacco use due to smoke alkalization and enhanced free-nicotine nicotine exposure*". In line with the conclusions of DIXON *et al.* (129), the authors questioned whether ammonia facilitated the pulmonary absorption per se (rate of absorption and total amount of nicotine absorbed) in view of the pH of the airways epithelium (133, 134) and the high buffering capacity of albumin, bicarbonate and ammonia in the lungs' lining fluid (130, 135, 136), which prevented tobacco smoke, including the ammonia in the smoke, from affecting the pH at the luminal side of the airways (137). Much rather, they were inclined to think that the enhancing effect of ammonia on nicotine absorption was confined to a concentration-driven increase in absorption due to the elevation of the free-base nicotine concentration in mainstream smoke. In conclusion, the authors called for additional studies on nicotine biomarkers in body fluids to provide proper, objective and independent scientific judgment about the effect of tobacco ammoniation on nicotine bioavailability.

SEEMAN (138) published in 2007 a comprehensive overview on the possible role of ammonia on the deposition, retention and absorption of nicotine in humans while smoking. Evaluating three of the four mechanisms of deposition described by PANKOW (122) the author examined the time dependent interaction of particulate ammonia and nicotine and emphasized the changes in composition occurring during the lifetime of the smoke particles, in particular the speedy loss of ammonia. Following deposition on the lung-blood interphases the acid-base status of the particles is quickly converted to the physiological pH, facilitating rapid nicotine absorption and overriding any possible ammonia manipulation. In the author's conclusion, determinations of "smoke pH" or the fraction of non-protonated nicotine in smoke particles under stationary conditions "*have little value, if any, in understanding, explaining, or predicting tobacco smoke chemistry or nicotine bioavailability from commercial cigarettes*" and "*the experimental data do not support any of the ammonia manipulation of nicotine hypotheses as they relate to commercial cigarettes*".

In a follow-up review, SEEMAN and CARCHMAN (139) expanded the survey of the possible effects of ammonia on the exposure, deposition and retention of nicotine during smoking and the bioavailability of nicotine to the smoker by addressing additional issues: Machine-smoking methods for the quantification of mainstream smoke nicotine and ammonia; absorption studies with charged nicotine analogues; the effect of ammonia on the absorption of nicotine from environmental tobacco smoke; the contribution of ammonia in cigarette smoke to human smoker toxicity; and a discussion of risk assessment. The authors confirmed that "*the experimental data indicate that neither nicotine transfer from tobacco to mainstream smoke nor nicotine bioavailability to the smoker increases with an increase in any of the following properties: tobacco soluble ammonia, mainstream smoke ammonia, "tobacco pH" or "smoke pH" at levels found in commercial cigarettes*".

In a recently published paper CHEN and PANKOW (140) opened a new round in the discussion of ammonia in tobacco smoke and its effects on nicotine. They concluded "*that a thorough examination of unbound and bound ammonia in mainstream tobacco smoke will be required before the role of ammonia in affecting volatility of nicotine in mainstream tobacco smoke can be understood*". The study of CHEN and PANKOW and their conclusions were heavily criticized by LAUTERBACH (141) primarily for the use of equipment unsuitable for the correct machine smoking of cigarettes according to ISO (73) or FTC (142)

and the MASSACHUSETTS DEPARTMENT OF PUBLIC HEALTH method (143), resp. LAUTERBACH pointed out that, for this reason, the CHEN and PANKOW'S data were so compromised as to render any conclusions drawn from them to be highly suspect. In their rebuttal, CHEN and PANKOW (144) confirmed two errata in their paper concerning the butt length and type of cigarettes smoked. In our opinion, their response to the other objections of LAUTERBACH (conditioning of the cigarettes before testing, smoking device, upward air flow across the cigarettes during smoking, etc.) is intricate and not convincing and does not allow other researchers to repeat the study.

In response to the appeal of WILLEMS *et al.* (132), VAN AMSTERDAM *et al.* (145) investigated in 2011 the impact of ammonia on nicotine absorption in smokers. In a controlled cross-over study 51 subjects smoked on two occasions two commercial filter cigarettes comparable in smoke nicotine, "tar" and carbon monoxide levels but different (by a factor of 3.8) in the ammonia content of the tobacco. It was shown that smoking the brand with the higher ammonia content did not result in higher serum nicotine levels in the smokers. There was also no significant difference with regard to blood pressure and heart rate. The authors concluded that "*enrichment of tobacco with ammonium salts does not lead to higher nicotine absorption and higher exposure of the smoker to nicotine*".

In summary, it can be concluded that the addition of ammonium compounds to tobacco under commercial conditions is no effective approach to increasing the "pH" of mainstream cigarette smoke or elevating the nicotine content of the smoke (146). It does not enhance nicotine absorption in the lungs and physiological nicotine impact. Whenever ammonium compounds are used as additives in cigarette manufacturing, it is for three reasons: First, for reacting with the sugars in tobacco to form valuable flavor substances; second, as processing agents in the production of certain types of reconstituted tobacco (147); and third in cigarette papers as combustion modifiers (148), for improving ash formation (149) and for reducing sidestream smoke emissions (150, 151) - this being the case with diammonium hydrogen phosphate in particular. In the production of expanded tobacco, ammonia and carbon dioxide are used as expansion agents (49).

3.3. Tobacco additives that are alleged to increase nicotine addictiveness

DENOBLE and MELE of Philip Morris, Richmond, VA (152) demonstrated in the early 1980s that both nicotine and acetaldehyde had positive reinforcing effects when administered intravenously to rats. When nicotine and acetaldehyde were applied together the effects in self-administering rats were not simply additive but synergistic (called "superadditive" by the authors). Consequently, it was assumed that acetaldehyde enhanced the addictiveness of nicotine.

It had been shown in a number of studies in 1969 and 1971 (153, 154) that aldehydes in cigarette mainstream smoke were mainly breakdown products of carbohydrates, e.g., sugars and cellulose. Specifically, this was investigated in the case of acetaldehyde in cigarette mainstream smoke by FENNER and BRAVEN (155). Regarding biological effects in smokers, DENOBLE and MELE'S hypothesis was questioned by POMERLEAU (156), who noted that the inhalation of cigarette mainstream smoke by humans did not ensure the delivery of acetaldehyde to the systemic circulation and that the amount of acetaldehyde generated by cigarette smoking "*seems too low to contribute much to the reinforcement of smoking*".

Even so, the observations of DENOBLE and MELE (152) together with the fact that sugars are used as additives in tobacco processing formed the basis for allegations that the tobacco industry added sugars to tobacco to increase the addictiveness of smoking (17, 157).

Acetaldehyde itself probably is not a natural tobacco component. It has been reported in low levels in tobacco (158) but this finding was not confirmed by others. In processed cased and flavored tobacco it may be present at low ppm levels as a component of some fruit and spice extracts used as flavorants (159–161) - and not as a separate additive.

In the processed cut tobacco of American blend cigarettes the total level of sugars (monosaccharides such as glucose and fructose, and disaccharides such as sucrose) may be as high as 20% (162). They originate from the natural sugars in the Virginia and Oriental tobacco moiety of the blend (about 50–70%) and the sugars added during processing. During smoking, nearly all carbohydrates are burned or decompose by pyrolysis.

More than 98% of acetaldehyde is present in the gaseous phase of cigarette mainstream smoke (163). The influence of the cigarette tobacco blend on mainstream smoke acetaldehyde yields was shown in 1962 by PAILER *et al.* (164). The yield of cigarettes made from Oriental tobaccos was five times higher than of cigarettes manufactured from dark, air cured tobaccos. Acetaldehyde levels between 0.234 and 0.916 mg/cig were determined in U.S. cigarettes for the mainstream smoke "tar" range of 0.7–17.2 mg (165). In the animal studies showing acetaldehyde acting as a positive reinforcing agent in the self-administration of drugs the levels of acetaldehyde were extremely high. Doses of 10 mg/kg body weight were used (166). For a 70 kg person, this is equal to the concurrent uptake of the mainstream smoke of 700 cigarettes (167).

During smoking the gas phase mainstream smoke component, acetaldehyde, is absorbed to 45–70% in the mouth and the upper airways (168) and rapidly metabolized to acetate by aldehyde dehydrogenase (169). Due to the very short half-life of a few seconds in biological environments no increase of acetaldehyde levels was observed in the blood of smokers (170). This limits the accumulation of acetaldehyde in the systemic circulation, and there is little chance for smoking derived acetaldehyde to actually reach the brain and overcome the blood-brain barrier (167). Therefore, synergistic effects in the central nervous system with nicotine taken up during smoking and the increase of its addictive potential seem quite unlikely.

In 2005, BELLUZI *et al.* (171) investigated the acquisition of nicotine self-administration in adolescent and adult rats. It was shown that acetaldehyde enhanced the acquisition of nicotine self-administration. The enhancement appeared to be synergistic but the absence of a full dose-response relationship precluded the definitive demonstration of synergy. Acetaldehyde enhancement was seen only in

adolescent rats and not in adults tested under the same conditions.

A review by TALHOUT *et al.* (172) in 2007 evaluated the presumed contribution of acetaldehyde to tobacco smoke addiction. They concluded that acetaldehyde in tobacco smoke may possibly increase the addictive potential of tobacco products by the formation of acetaldehyde-biogenic amine (tryptamine) adducts (e.g., harmane) in the cigarette smoke and/or *in vivo*, which may inhibit monoamino oxidase in smokers. Because the signs of addictiveness due to the synergistic action of nicotine and acetaldehyde were only observed in young but not in adult rats, TALHOUT *et al.* went so far as to speculate that this might be critical for initiating smoking in young people. However, additional research was called for to substantiate the hypothesis.

In 2002, SEEMAN *et al.*(167) published a literature review on the formation and occurrence of acetaldehyde in cigarette mainstream smoke and its bioavailability to the smoker. They concluded after evaluating the published literature that there was no relationship between simple sugars in tobacco and the amount of acetaldehyde in mainstream smoke. This indicated that sugars (monosaccharides and disaccharides) were not the main precursors for acetaldehyde in tobacco smoke. The extraction of sugars from tobacco did not result in less acetaldehyde being formed during smoking (173). The ^{14}C-label in glucose or sucrose was recovered in smoke only to 0.05–0.06% in the form of acetaldehyde (153). In a long term study, SEEMAN *et al.* (174) examined the relationship between cigarette mainstream smoke acetaldehyde, carbon monoxide and "tar" and the percentage of total reducing sugars in the tobacco blend. With time, 2,114 commercial cigarette brands were analyzed, which were produced between 1985 and 1993. Mainstream smoke acetaldehyde was significantly correlated with "tar" and carbon monoxide but not with the concentration of reducing sugars in the tobacco blends. These results supported the hypothesis that cigarette mainstream smoke acetaldehyde yields were more affected by cigarette design characteristics influencing total smoke production than by the reducing sugars in tobacco. The primary precursors in tobacco for the formation of acetaldehyde are the structural polysaccharides of the tobacco leaves and ribs, such as cellulose, hemicelluloses and pectin, as well as starch (173, 175).

The German regulatory authorities initiated a research project to gather more information concerning the influence of tobacco additives on the composition of cigarette mainstream smoke (176). The study investigated the effects of the tobacco additives, sucrose, cocoa and glycerol, on selected mainstream smoke constituents of two types of cigarettes with identical tobacco blend but different mainstream smoke "tar" and nicotine levels. The test cigarettes were machine made and filter ventilated; their tobacco was a typical American blend. In the mainstream smoke of the two types of cigarettes the addition of sucrose to the tobacco blend did not lead to an increase in acetaldehyde yields. The results of this study were confirmed by laboratories of the cigarette industry (177, 178).

In a recent study, CAHOURS *et al.* (179, 180) did not see a correlation between the sugar content of cigarette tobacco and the acetaldehyde level in mainstream smoke. This had also been pointed out by BAKER in 2007 (181).

3.4. Attractiveness

In the current debate on the regulation of additives, the concept of attractiveness is gathering more and more attention from regulators. According to the WHO, the terms "attractiveness" or "consumer appeal" refer to factors such as taste, smell and other sensory attributes, ease of use, flexibility of the dosing system, cost, reputation or image, assumed risks and benefits, and other characteristics of a product designed to stimulate use. The importance of the attractiveness of tobacco products for their addictiveness and for abuse liability was discussed in 2010 at two conferences on the appeal of tobacco products and abuse liability (182, 183).

The attractiveness of a product is an important factor in a market economy and a legitimate goal for products in a competing market. The concept of regulating products with the objective of reducing their attractiveness is truly irritating.

Like other industries, tobacco companies are part of the open market and compete with other companies by developing and differentiating their products. Additives are an important part of the manufacturing process in general but in American blend cigarettes additives play also a vital role for product integrity and consistency. They are used to create a particular taste and flavor signature for specific brands and differentiate the product in the market place.

Therefore, it is the natural goal of adding aromatic substances to tobacco during cigarette manufacturing to enhance the attractiveness of the brand in comparison to competitors' products on the market.

In 2010, the EU Scientific Committee on Emerging Newly Identified Health Risk (SCENIHR) came to the following conclusions: "*Attractiveness depends on multiple factors that combine to stimulate use. These include extrinsic factors such as marketing, packaging and price, and intrinsic factors such as taste and smell. It is very difficult to identify the role of individual additives in enhancing addictiveness or enhancing other attractive attributes of tobacco products.*" (92, on page 73).

"*In conclusion, many different additives have been used to increase the attractiveness of tobacco products but it is very difficult to identify the role of individual additives in enhancing attractiveness. ... Animal models do not currently exist to allow the assessment of attractiveness. ... However, such studies in human subjects are difficult to carry out due to ethical considerations and the current methods are thus not considered adequate for a reliable quantification of attractiveness in humans*." (92, on page 85-86).

A scientifically valid and convincing approach to evaluating the influence of flavorings on the attractiveness of smoking is not available up to now. If the concept of attractiveness was applied to the regulation of additives, there would be a requirement to develop a clear scientific basis.

In consideration of the "unworkable" concept and the lack of methods and data, claims of enhancing product attractiveness are not intensively discussed for individual additives in the following chapters.

3.5. *The consumer preference for American blend cigarettes vs. Virginia cigarettes*

It is particularly worth to mention the following example of the pitfalls of reflections that additives in general or some of them are responsible for the attractiveness and addictiveness of tobacco products. As pointed out above, the addition of flavorings and casings improves and determines the distinct smoke taste and aroma of American blend cigarettes. On the other side, cigarettes made from pure Virginia, dark air cured or Orient tobaccos usually contain no additives (specifically no flavorings) and are nevertheless successful in several markets.

Looking at today`s (2008) cigarette markets (10), American blend cigarettes are largely preferred in continental West European countries holding a market share of 85–100%. Virginia style cigarettes dominate the market in countries such as Australia (92%), Canada (99%), Ireland (87%), New Zealand (95%), South Africa (76%) and the United Kingdom (91%). Not surprisingly, American blend cigarettes dominate the market in the United States with constantly over 99% (184).

The divergence in consumer preference is not similarly reflected by the data (available for 2010) on smoking prevalence (185), which is not markedly different in American blend countries (France: 33%; Germany: 25%; Italy: 26%; the Netherlands: 24%) from Virginia style markets (Ireland: 31%; United Kingdom: 28%).

Such statistical data are an indication that aromatic additives in cigarettes have no clear-cut influence on smoking prevalence and do not strongly affect the tendency of young people to start smoking.

SANDERS *et al.* (186) took an interesting approach for evaluating the effect of cigarette additives on addictiveness. By meta-analysis they compared the cessation rates of smokers of cased and flavored American blend cigarettes to those containing few or no additives (Virginia cigarettes). The data were obtained in randomized clinical studies evaluating the effectiveness of nicotine replacement therapy in smoking cessation. 108 data sets from different studies published between 1980 and 2012 were included. 20 data sets originated from countries where primarily cigarettes were consumed with no, or minimal amounts of, additives (United Kingdom, Australia, New Zealand, Canada). 88 data sets were from countries where primarily American blend cigarettes were smoked (U.S.A., Sweden, Denmark, Croatia, Belgium, Iceland, Italy, Spain, Switzerland, the Czech Republic, Finland, France, the Netherlands). The main conclusion of this analysis was that there was no evidence of the cessation rates of smokers of cased and flavored cigarettes being lower than those of smokers of additive free Virginia cigarettes. On the contrary, there was a small but statistically significant difference that favored cessation in smokers of American blend cigarettes. The authors were not inclined to consider this result a real effect as there was no rational explanation suggesting that the presence of casing and flavoring substances in cigarettes might increase the ease of smoking cessation. "*However, all the data are consistent with the conclusion that the presence of additives currently being added to tobacco does not increase the inherent cigarette addictiveness*".

3.6. *Overflavored products*

Classical cased and flavored American blend cigarettes have typical tobacco taste and aroma based on the blend of Virginia, Burley and Oriental tobaccos. However, this is not necessarily true for cigarettes with strong taste components other than tobacco, such as lime, honey, cherry or strawberry, which were recently introduced into the market (187). It is assumed by health advocates that flavored cigarettes of this kind are particularly attractive and may be a way to introduce adolescents to smoking (188, 189). These "sweet", "spicy" or "fruity" cigarettes cannot be regarded as American blend cigarettes and are not likely to be accepted by habitual smokers as shown by KLEIN *et al.* (190).

4. COMPOSITION AND TOXICITY OF CIGARETTE MAINSTREAM SMOKE WITH AND WITHOUT ADDITIVES

In view of the widespread use of additives in cigarette manufacturing it is of paramount importance to assess their safety for consumers. The in-depth analysis of the effects of additives on the composition and the *in vitro* and *in vivo* toxicological activity of mainstream smoke is essential for meaningful conclusions and responsible judgment. It is a sound approach taking guidance from the wealth of data available for chemical compounds and preparations used as food additives and to expand the knowledge base by additional specific research focused on the fate of additives in consumer products intended for smoking.

Looking at the situation in the Unites States, an important characteristic of food additives is their status as "generally recognized as safe" (GRAS). The status is allocated to "*substances that are generally recognized, among experts qualified by scientific training and experience to evaluate their safety, as having been adequately shown ... to be safe under the conditions of their intended use*" (191). The determination that an additive is GRAS may be made by any qualified group of experts outside of government. An important role in this is played by the Flavor and Extract Manufacturers Association (FEMA). Since 1960, an Expert Panel of FEMA is responsible for the safety evaluation of food flavorings pursuant to the authority granted in Section 201(s) of the U.S. Federal Food, Drug, and Cosmetic Act. Lists of additives with GRAS status have been published by FEMA regularly since 1965, making them accepted as safe food ingredients for their intended use in many countries around the world.

The 1958 Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act and supporting legislative documents established the rules and procedures for the regulation of food additives by the U.S. Food and Drug Administration (FDA). The GRAS status of substances may also be affirmed by the FDA on the basis of qualified (outside) expert opinion. Formalities of the process were modified several times in the past. Substances without GRAS status must undergo a premarket evaluation process by the FDA under 21 CFR 171.1, resulting (if successful) in the status of "FDA approved food additive".

It provides a realistic perspective to know that a large proportion of the tobacco additives currently used in cigarette manufacturing (more than 95%) has FEMA GRAS and/or FDA GRAS status, and/or is an FDA approved food additive.

GRAS status or FDA approval are valuable starting points for assessing the safety of a tobacco additive. However, additional targeted considerations are mandatory for deciding how far this carries under the conditions of smoking. In the first place, it is necessary to know whether a tobacco additive is actually subjected to thermal degradation and/or combustion during smoking - and if so, to which extent. Research has shown that a high proportion of tobacco additives escape the burning zone largely or completely intact and show up unchanged in mainstream smoke. If the fate of an additive includes thermal degradation and/or combustion then the nature and amounts of the substances formed during smoking have to be investigated. Well designed pyrolysis studies are the approach of choice for the clarification of these questions.

4.1. Pyrolysis studies

A large amount of research has been performed on tobacco pyrolysis and combustion, two main chemical processes occurring during tobacco smoking, and their physical conditions. Pyrolysis is the endothermic decomposition of organic materials at elevated temperatures. During smoking, rise in temperature is an effect of combustion, an exothermic self-sustaining chemical reaction.

The identification of precursors for smoke constituents and the possibilities of influencing smoke composition are two pertinent areas of investigation. To understand the effects of tobacco additives on the composition and toxicity of cigarette mainstream smoke it is essential to know how they behave under the conditions of smoking: Whether and to which degree they are transferred intact to the smoke, and whether there is any decomposition to pyrolysis (or formation of pyrosynthesis) products, which become components of mainstream smoke. It must be known whether additives result in new compounds or higher concentrations of hazardous components in the smoke. If this is the case, knowledge about their nature and amount is mandatory. This is to say that well designed and realistically performed pyrolysis studies are the first steps in the toxicological assessment of tobacco additives.

In the mid 1950s, WYNDER and HOFFMANN (13, 192) suggested that the behavior of a compound during pyrolysis would be equivalent to its fate in tobacco during smoking. Already in the 1960s, first studies were done looking at the fate of certain materials added to tobacco during the cigarette manufacturing process, and very many followed in the course of the last decades. Over the years hundreds of papers on the pyrolysis of tobacco, tobacco constituents and tobacco additives were published. The design and means of the studies are rather varied. As we know today, experimental approaches were often scientifically and technically quite inappropriate but the data produced and (unreasonable) conclusions drawn linger on in the scientific literature and public debate.

The problem is impressively illustrated by the study of SCHMELTZ *et al.* (193) on nicotine pyrolysis. ^{14}C-labeled nicotine was adsorbed onto silica gel or mixed with tobacco and isothermally pyrolyzed in a combustion tube under nitrogen at temperatures between 600 °C and 900 °C. Under these pyrolysis conditions nicotine underwent degradation to pyridines by simple bond cleavage as well as rearrangement to quinolines, arylnitriles and even aromatic hydrocarbons. However, when cigarettes were smoked containing labeled nicotine, a substantial portion (about 42%) distilled intact into mainstream and sidestream smoke. Up to 11% was converted to pyridines by simple degradation but no significant amounts of quinolines, arylnitriles or aromatic hydrocarbons were found. In addition, 12.5% of the nicotine was oxidized to carbon dioxide. Obviously, under the chosen conditions of the pyrolysis study thermal degradation had occurred far more extensively than observed in a burning cigarette.

For conducting a meaningful pyrolysis study, the full understanding of the dynamic physical and chemical processes in the burning zone of a cigarette is indispensable. The fate of an additive during smoking can be sensibly simulated only if the pyrolysis conditions, such as temperature, heating rate, oxygen level and gas flow parameters, are sufficiently close to those occurring during smoking. These conditions vary considerably within a cigarette depending on the position relative to the burning cone. They had been investigated and reasoned out with great sophistication for some time (194).

From a different viewpoint the crucial influence of pyrolysis conditions, especially temperature, was shown by WHITE *et al.* (195) when they assessed the mutagenicity of the condensate of tobacco smoke aerosols, which were generated under precisely controlled temperature conditions from 250 °C to 550 °C by heating compressed tobacco tablets in air.

- STOTESBURY *et al.* (196, 197)

With keen eyes on the conditions in the burning or smoldering cigarette cone STOTESBURY *et al.* (196) defined the technical requirements for conducting a meaningful study with six neat model tobacco additives, namely the flavorants anisole, *p*-anisaldehyde, benzaldehyde, isoamyl isovalerate, methyl *trans*-cinnamate and vanillin. For each test, a small sample was collected on quartz wool in a capillary tube and subjected for 3 sec to a range of set temperatures (200–900 °C) and atmospheres (2% and 10% oxygen in nitrogen) in a pyrolysis apparatus connected to a GC/MS. The additive behavior in relation to temperature showed increasing volatilization and intact transfer with rising temperatures and the concurrent formation of degradation products at higher temperatures, which was, however, impressive only in the case of benzaldehyde. With a view at cigarette smoking, it was predicted from the data that anisole, isoamyl isovalerate and vanillin would be transferred (97% or more) intact to smoke at 200 °C, *p*-anisaldehyde and methyl *trans*-cinnamate (also 97% or more) at around 400 °C with some decomposition of 1–3%, and benzaldehyde (74%) from 200 °C on, but at this temperature already with significant oxidation to benzoic acid. These forecasts were well in line with the data obtained in a study by GREEN *et al.* (198), who had analyzed the smoke of experimental cigarettes spiked with ^{14}C-

labeled samples of the same flavor additives as used by STOTESBURY *et al.* (196).

However, there was a discrepancy regarding the degree of intact transfer of *p*-anisaldehyde and vanillin. This was resolved in another study by STOTESBURY *et al.* (197), who examined the two additives labeled with the stable isotopes, ^{13}C and ^{18}O, in an experimental setup similar to the one of the first study (196). The low amount of degradation by pyrolysis (less than 1%) and the expected high intact transfer values (nearly 100%) were confirmed.

The work of STOTESBURY *et al.* (196, 197) illustrates the scientific potential of, and imperative careful deliberations required for, conducting pyrolysis studies. On this basis, a sub-group of the U.K. Tobacco Manufacturers' Association Additives Working Party considered the pyrolysis of tobacco additives in 1999 and 2000. Their objective was to establish a set of pyrolysis conditions that could be used to predict the pyrolytic behavior of tobacco additives during smoking. Following collaborative studies, non-isothermal pyrolysis was viewed as most suitable and suggestions were made concerning sample size, atmosphere, temperature and exposure time. These parameters were drawn on by BAKER and BISHOP in the pyrolysis studies discussed next.

- BAKER and BISHOP (199, 200)

Based on their profound understanding of the dynamic physical and chemical processes in the burning zone of a cigarette (163) and following the critical evaluation of many relevant papers on the pyrolysis of tobacco, tobacco constituents and tobacco additives published or presented at conferences, BAKER and BISHOP (199) stipulated a regimen for conducting meaningful pyrolysis studies with neat single compounds used as tobacco additives. The essential component of the equipment used was a pyrolyzer coupled to a gas chromatograph/mass spectrometer. The sample (200 µg, in ethanol solution or in solid form) was deposited on quartz wool and heated inside a small quartz tube under a flow of 9% oxygen in nitrogen (at 4.6 mL/sec) using the following conditions: holding the initial temperature of 300 °C for 5 sec, increasing the temperature from 300 °C to 900 °C with a ramp of 30 °C/sec; holding the final temperature of 900 °C for 5 sec. The rationale behind each parameter was the best possible approximation of the temperature conditions in the glowing cone during cigarette smoking, which were described by MURAMATSU (201) and BAKER (202, 203).

Smoking studies with cigarettes containing the substances under investigation in labeled form (using ^{14}C and also the stable isotopes ^{13}C, ^{18}O and deuterium) are the most appropriate and definitive way of determining the intact transfer of an additive to mainstream smoke and the potential formation of pyrolysis products. BAKER and BISHOP (199) identified thirteen relevant published studies of this nature, which had examined the transfer of eleven labeled compounds (and the pyrolytic decomposition of five of them). For appraising whether the pyrolysis conditions they had designed were realistic BAKER and BISHOP applied their technique to these eleven compounds and compared the results to those of the labeled studies.

For nine of the eleven additives with considerable volatility, which were transferred > 95% intact, the two types of studies showed a high degree of agreement. Two substances with lesser volatility were transferred intact to mainstream smoke in the smoking studies at a clearly higher rate than in the pyrolysis experiment. It was reasoned that specific events in a burning cigarette, such as elution (displacement of a substance from the tobacco and trapping in aerosol particles), might intensify the intact transfer over what could be observed in experimental pyrolysis. Where the (generally minor) pyrolysis products were analyzed in the labeled smoking studies, their pattern was essentially similar, but not identical to that seen in the pyrolysis trials.

The comparison, as described, of the data from labeled smoking studies and the pyrolysis experiments showed that "*the pyrolysis system developed gives good predictions of the smoke transfer/pyrolytic behaviour of relatively volatile tobacco [additives]. For involatile substances it gives an over-estimation of the amount of pyrolytic decomposition that occurs in the burning cigarette*" (199).

Next, 291 single tobacco additives were subjected to pyrolysis as described with the objective of estimating the degree of intact transfer to mainstream smoke and analyzing qualitatively and quantitatively the formation of pyrolytic products whenever decomposition occurred. Detailed data were compiled in a large table showing the name, CAS number, formula or structure, chemical class, and molecular weight of the additive, its boiling and/or melting points, the maximal recommended inclusion level when used in commercial cigarettes manufactured by British American Tobacco, the purity of the sample pyrolyzed, the composition of the pyrolysate with percentages (including the amount transferred intact), and the maximum amount (µg/cig) of each component in the pyrolysate predicted to be found in the mainstream smoke of a plain cigarette (calculated on the basis of several worst-case assumptions). Where decomposition occurred, all (or at least the ten major) pyrolysis products were listed. A threshold of toxicological concern of 0.03 µg/cig was presumed adopting thinking applicable to untested chemicals in food - for details see (199).

It was determined that 92 of the tobacco additives (almost one third) transferred ≥ 99% intact out of the pyrolysis zone (less than 1% pyrolysis) and 184 additives (over 63%) transferred ≥ 95% intact (less than 5% pyrolysis).

Looking at the products of pyrolysis, particular attention was directed at the so-called "Hoffmann analytes", a compilation of 44 substances believed by regulatory authorities in the United States (204) and Canada (205) to have impact on smoking related diseases. Included in this assortment of compounds are some volatile carbonyls, tobacco specific *N*-nitrosamines, aromatic amines, phenols, volatile alkenes, benzo[*a*]pyrene and metals. The list had been developed since the mid 1980s by Dietrich Hoffmann of the American Health Foundation and had grown to 82 "*biologically active agents in the mainstream smoke of nonfilter cigarettes*" by 2001 (206).

Based on the measurements of pyrolysis products, a total of seven of the 44 core "Hoffmann analytes" were found in the pyrolysate of 19 tested additives at predicted levels of ≥ 0.03 µg/cig. These were mostly phenol and styrene, and in one case each acetaldehyde, butanal, benzene, toluene and cresol. Generally, their predicted mainstream smoke

levels were minute fractions of the typical amounts in the smoke of an unfiltered cigarette. In conclusion, the number of Hoffmann analytes detected in the pyrolysates of the 291 additives was rather small and their level distinctly low.

In the second part of their systematic pyrolysis study BAKER and BISHOP (200) turned their attention to a further 159 complex, non-volatile tobacco additives and additive mixtures, applying the technique used before (199) with the 291 single, mostly semi-volatile substances. Similarly, a large table was developed listing for each material the name and CAS number, the maximum recommended inclusion level in commercial cigarettes, the composition of the pyrolysate with percentages, and the maximum amount (μg/cig) of each component in the pyrolysate predicted to be found in mainstream smoke (again calculated on the basis of several worst-case assumptions). Most, possibly all, additives decomposed completely in the assays; the five most abundant pyrolysis products were included in the table together with any "Hoffmann analytes" detected.

With many of the test materials no "Hoffmann analytes" were found in the pyrolysates. However, 56 of the 159 additives did produce "Hoffmann analytes" (mainly phenols, benzene, toluene or styrene) or furfural (a biologically active compound, though by coincidence not a "Hoffmann analyte") in amounts predicted to be as high as ≥ 0.03 μg/cig, which for some additives were small compared to those found in the mainstream smoke of plain cigarettes but for other additives higher, especially with regard to furfural produced by the pyrolysis of carbohydrate materials.

The insight gained in the earlier study (199) that the pyrolytic decomposition of poorly volatile substances may be overestimated under the chosen test conditions was confirmed. It seems that the additives are subjected to stronger thermal force in the experimental setup than in a burning cigarette. This effect is likely in particular for the pyrolytic formation of furfural from sugars.

In these cases, experimental pyrolysis must be considered as the first step followed by further assessment in smoking studies with the additives of concern incorporated in cigarette tobacco. Exactly this was done with the 56 additives shown to be capable, when pyrolyzed, of producing compounds of toxicological concern. This study was reported elsewhere (207, 208) and is discussed below (see Section 4.4. on page 431).

- PURKIS, MUELLER and INTORP (209)

Recently, PURKIS *et al.* (209) published the results of several studies looking into the fate of tobacco additives under various experimental conditions. The main item was the pyrolysis of 91 semi-volatile tobacco additives using a technique very similar to, but not identical with, the one used by BAKER and BISHOP (199). Most of the compounds showed high levels of intact transfer into the pyrolysate: 100% transfer was determined for 50 substances (55%), and ≥ 90% transfer in the aggregate for 80 additives (88%). No consistent relationship was observed between intact transfer and molecular weight or boiling point. It seemed that lower volatility and high polarity (like with acids) were not conducive to intact transfer, while multiple double bonds (like in geranyl acetate) promoted molecular rearrangement.

In order to assess the relevance of pyrolysis for cigarette smoking, the authors conducted a series of additional experiments. Tests with two deuterated additives were particularly informative. When neat benzaldehyde-d_6 was pyrolyzed mimicking conditions in a cigarette (temperature program from 300–900 °C with a ramping rate at 25 °C/sec and 2% O_2 in N_2 gas flow through the pyrolysis tube), 97.9% was recovered intact together with the only degradation product, benzene-d_6 (2.1%). Harsher pyrolysis conditions (isothermal at 900 °C) produced 26.1% benzene-d_6 and 2.6% biphenyl-d_{10}. Pyrolysis of an experimental mixture of benzaldehyde-d_6 and tobacco yielded 0.12% benzene-d_5, 0.83% toluene-d_5 and 0.24% methyl biphenyl-d_9. However, the smoking of a cigarette with identical tobacco and comparable benzaldehyde spiking allowed the recovery of 72% benzaldehyde-d_6 in mainstream smoke, sidestream smoke, butt and ash combined but none of the decomposition products mentioned above. Similarly, phenylacetic acid-d_7, which in the pyrolysis test was transferred 91.7% intact along with five degradation products, produced only a small amount of toluene-d_7 in the mainstream smoke of a spiked cigarette. These results illustrate the degree of caution required for conducting and interpreting pyrolysis studies.

Cigarette mass balance studies were carried out with 11 semi-volatile unlabeled additives of different chemical classes to determine the occurrence of intact compounds in mainstream smoke, sidestream smoke, and but and ash combined. Total intact recoveries ranged from 32% (for ethyl vanillin) to 90% (for ambroxan).

Particularly informative were the mass balance studies with seven ^{13}C-labeled additives (vanillin, glucose, benzaldehyde, glycerol, 1,2-propylene glycol, tetramethyl pyrazine and geranyl acetate), which included the measurement of small molecules and gases. It was concluded that substantial amounts of products of incomplete combustion were not present in mainstream smoke and the materials not remaining intact were primarily converted to simple products, such as carbon monoxide and carbon dioxide.

The authors pointed out that the results of their experiments demonstrated that pyrolysis studies were useful for distinguishing between the substances being transferred intact into mainstream cigarette smoke and those liable to degrade, but did not provide robust predictions of the compounds formed from additives during cigarette smoking.

4.2. Toxicity testing in vitro and in vivo

Toxicity testing is a key element in the safety assessment of consumer products such as cigarettes. Meaningful tests may be performed *in vitro* and *in vivo*. Numerous approaches and methods are in use and described in the scientific and regulatory literature. A comprehensive review of toxicological *in vitro* methods and their use in strategies for characterizing and predicting hazards to humans - though without special consideration of tobacco issues but with attention to the development and application of biomarkers for the elucidation of cancer risks - was published in 2002 by EISENBRAND *et al.* (210).

Turning to smoking products, prudent deliberations are called for with the focus on the specific conditions and

requirements of testing tobacco, tobacco additives and finished cigarettes. The challenge was taken on by a CORESTA TASK FORCE, who produced a report in 2004 on "*the rationale and strategy for conducting in vitro toxicology testing of tobacco smoke*" (211). Key procedures were identified based upon internationally recognized guidelines and adapted to accommodate the nature and unique properties of tobacco smoke. At the same time, the report did not provide interpretational guidance concerning the biological significance of *in vitro* results. For sound assessment, all available chemical and biological data must as well be considered and brought into accord with the features of the tested material in a weight-of-evidence approach.

Most methods of *in vitro* testing are rapid and relatively inexpensive. They provide a quantitative, mechanistically based measure of biological response. For evaluating the toxicity of whole tobacco smoke or its fractions (gas phase and particulate phase = condensate) the Task Force recommended the following battery of assays suitable for determining cytotoxicity (effects on cell viability and growth rates) and genotoxicity (DNA damage):

- A mammalian cell line cytotoxicity assay, specifically the neutral red assay. It measures the trapping of a weakly cationic dye by lysosomes, which is impaired or stopped in damaged or dead cells.
- A bacterial mutagenicity assay, specifically the Ames assay. It uses different strains of *Salmonella typhimurium* that have been modified to be extremely sensitive to mutagens, and measures strain-specific reverse point mutations. To make up for the metabolic capabilities of mammals (missing in bacteria) an exogenous activation system is generally used for the conversion of promutagens to mutagens. It consists of a tissue homogenate and cofactor mix - most commonly the so-called S-9 mix prepared from rat liver.
- A mammalian genotoxicity assay. Options are the micronucleus assay, which identifies small membrane-bound bodies containing chromosome fragments or whole chromosomes that are unable to migrate during cell division; the chromosome aberration assay measuring structural changes and rearrangements in chromosomes resulting from DNA damage, which, however, are difficult to score; and the mouse lymphoma assay (MLA), which - depending on the protocol - is able to detect a rather wide range of genetic damage.

Combinations of these assays were considered to provide a sensible foundation for assessing the *in vitro* toxicity of tobacco smoke and its fractions. Procedures specifically adapted to tobacco smoke (including smoke preparation and data analysis) for performing the neutral red assay, the Ames assay and the micronucleus assay on tobacco smoke were developed and described by the Task Force.

The conclusions reached by ANDREOLI *et al.* (212) in their review of toxicological *in vitro* methods for the assessment of the biological activity of tobacco smoke are very much in line with those of the CORESTA TASK FORCE report (211). They recommended the neutral red uptake assay as cytotoxicity test, and the Ames *Salmonella* and micronucleus assays for measuring genotoxicity.

The extensive evaluation of *in vitro* assays for assessing the toxicity of cigarette smoke and smokeless tobacco by JOHNSON *et al.* (213) identified tobacco related toxicological *in vitro* studies published since 1980 (and a few earlier ones with high relevance) and compiled both methodological details and reported results in several large tables. The qualitative reliability of *in vitro* assays as screening tools, their validation for quantitative comparisons of different tobacco products and any extrapolations of *in vitro* data to human risks were critically reviewed.

Animal inhalation studies represent an important approach to the *in vivo* characterization of tobacco smoke toxicity. In recent years, the examination of novel cigarette designs (214) and the safety assessment of tobacco additives were major inducements for performing such studies. Rats (mostly Sprague-Dawley) and mice were the preferred experimental animals (215) but other species were also used, such as hamsters (nearly always Syrian Golden hamsters), dogs and nonhuman primates (216). Unlike *in vitro* assays inhalation studies in general are technically rather demanding, time consuming and expensive. They are performed with the objective of reflecting the conditions and consequences of (human) smoking in a more realistic way than carried through in *in vitro* studies.

The experimental animals are exposed to the test materials either nose-only in conical constraint tubes or whole-body in larger chambers. Usually, sham controls (animals exposed under identical conditions to air only) and cage controls are included. Exposure intensity is determined by the exposure time per day, exposure days per week, the duration of the study (14 days or 90 days in subchronic studies, up to over 2 years in chronic studies, occasionally even for lifetime) and the concentration and composition of the smoke. The generation of smoke, its analytical characterization and the distribution to the animals are major technical challenges. An important parameter to know is the amount of smoke actually inhaled and taken up by the animals. This can be assessed by measuring carboxyhemoglobin and plasma nicotine and cotinine, whereby the level of carboxyhemoglobin in the animals' blood determines the upper limit of smoke exposure and is being controlled for avoiding intoxitation.

The effects of smoke exposure are evaluated in a battery of examinations that may be rather complex: gross observations, viability, body weights and food consumption, respiratory physiology, blood chemistry, necropsy and gross pathology, and - above all - histopathology. The main inspection criteria for assessing smoke effects are epithelial hypertrophy, hyperplasia and squamous metaplasia in the conducting airways (visible in most studies), intra-alveolar macrophages and alveolar metaplasia, and lung adenomas and adenocarcinomas (seen in only a few studies).

Whether inhalation studies (with rodents) are in fact a useful model for the pulmonary carcinogenicity of cigarette smoke as it is observed in man remains a matter of debate. A tumorigenic response to cigarette smoke inhalation is either not seen at all or too weak for statistical toxicological assessment. Why inhalation studies generally do not reflect the findings of epidemiology is presently not well understood.

An animal test system, which uses the mouse strain A/J and where lung tumors can actually be produced, has been practiced and evaluated in depth by WITSCHI *et al.*(217). The assay uses a split protocol: Animals are first exposed whole-body for up to 5 months to a mixture of cigarette mainstream and sidestream smoke, and then allowed to

recover in air for another 4 months. Lung tumor multiplicities and (less importantly) incidence are then obtained by counting tumors visible on the surface, a procedure that is assumed to reflect the total tumor count per lung and to reveal the carcinogenic potential of the smoke. The tumor dose-response curve is not very steep, making cigarette smoke a rather weak mouse lung carcinogen. The strengths and weaknesses of the model are being discussed controversially (218); it has yet to reach the stage of practical applicability.

In a research study (219) male and female F344 rats were exposed whole-body to cigarette mainstream smoke for up to 30 months. The incidences of non-neoplastic and neoplastic proliferative lung lesions were significantly increased only in females, not in males. In another research study (220) with female B6C3F1 mice, near life-time whole-body exposure induced marked, exposure related increases in the incidence and multiplicity of hyperplastic, benign and malignant epithelial lesions in the lungs.

An alternative *in vivo* model for assessing the tumorigenic activity of cigarette smoke (in practice, only its condensate) is the mouse skin painting assay. It provides a fairly rapid response and allows the relatively easy quantification of tumorigenic potency in terms of tumor incidence, latency, multiplicity and malignancy (221). The use of the SENCAR mouse strain is well established, which is more sensitive than the B6C3F1 or Swiss CD-1 strains. Recently, the usefulness of the hairless (but not "nude") SKH-1 strain was demonstrated for the testing of cigarette smoke condensate (222). The skin painting assay employs a two-stage approach. First, a non-tumorigenic dose of a known mutagenic/carcinogenic substance is applied (once or only a few times) to the shaved or naked dorsal skin of the animals for tumor initiation, followed by repeated applications at the same site of the material to be tested for tumor promotion and progression. Over time, the initial formation of benign skin tumors is succeeded by the development of malign skin tumors. The skin painting test has been used in a number of studies, which are discussed in this review.

Attention: When evaluating published toxicological *in vitro* and *in vivo* data the methodological specifics and possible modifications should be carefully verified and compared in every case.

4.3. Literature reviews

In this section, the major published reviews of the literature on the effects of tobacco additives on cigarette mainstream smoke composition and toxicity are discussed.

- DOULL *et al.* (223)

In the mid 1980s, the major U.S. manufacturers had prepared individual lists of the additives used in cigarette production at this time. Subsequently, the lists of American Tobacco Co., Brown & Williamson Tobacco Corp., Ligget Group Inc., Lorillard Tobacco Co., Philip Morris Inc. and R.J. Reynolds Tobacco Co. were combined and submitted in 1986 to the Office of Smoking and Health of the U.S. Department of Health and Human Services - with an update each following year. The Office was legally required to review the list and report any concerns it had about the additives. No such report was ever made to Congress or the cigarette manufacturers.

At the request of the cigarette industry six prominent U.S. toxicologists, headed by J. DOULL of the University of Kansas Medical Center, examined the scientific data available for the listed additives extensively and independently, including confidential unpublished material of the six cigarette companies. Maximum use levels and annual poundage information, pyrolysis and transfer data, analytical reports, and results of toxicity tests were evaluated. In 1994, the most current, complete list of additives was made public combined with a summary of the conclusions of the six toxicologists (223). There were 599 additives on the list: 460 individual compounds and 139 mixtures, such as natural essential oils, plant and fruit extracts and oleoresins. The additives were compiled in alphabetical order together with their regulatory status (more than 98% were food additives approved by the U.S. Food and Drug Administration (FDA), and/or were "generally recognized as safe" (GRAS) by the FDA and/or the Flavor and Extract Manufacturers Association (FEMA)), their natural occurrence and their use in food products.

In their safety assessment, the six toxicologists observed that many of the additives reviewed were "*identical or essentially similar in composition to natural leaf tobacco components. The pyrolysis products of such ingredients are not expected to depart significantly from the amounts or types of components generated from a range of additive-free tobaccos or tobacco blends. Furthermore, the ingredients do not contribute measurably to tar yields*". Upon examination of "*extensive published and unpublished toxicologic, metabolic, and pyrolysis data*" the expert panel concluded "*that there was no evidence that any ingredient added to cigarette tobacco produces harmful effects*" and "*that the ingredients added to tobacco in the manufacture of cigarettes by the six major United States manufacturers are not hazardous under the conditions of use*".

- PASCHKE, SCHERER and HELLER (224)

In 2002, PASCHKE *et al.* (224) released a literature review based on published scientific studies of the effects of tobacco product ingredients and various experimental additives on tobacco smoke composition and biological activity. The format of this paper was that of an uncommented reference paper rather than a critical review. The mentioning of an additive in this review did not imply that it was actually used by the tobacco industry.

The review is a helpful overview of the information published up to 2002 on tobacco product ingredients, their transfer into mainstream smoke, pyrolysis products and influence on the composition and biological activity of cigarette mainstream smoke. Only those papers were considered that included a discussion of the influence of a tobacco additive on the composition and properties of mainstream smoke. Also regarded were studies, which investigated the pyrolysis products of additives or their mixtures. Studies dealing with materials in filters or tipping paper not expected to appear in mainstream smoke, such as cellulose acetate or non-volatile compounds and

salts, were excluded. Publications, which analyzed the toxicological effects of additives in unchanged or pyrolyzed form independent of their application on tobacco, were also not considered.

More than 1,000 additives were covered in the review in different tables. The tables indicated whether an additive had been investigated as a single compound or in a mixture. Information concerning its function in tobacco products and data on mainstream smoke were provided. Pyrolysis data and data concerning the influence of the additive on the biological activity of mainstream smoke were also presented.

PASCHKE *et al.* pointed out that most substances used as additives in commercial tobacco products were "generally recognized as safe" (GRAS) by the FDA for use in foods and/or listed on the Flavor and Extract Manufacturers Association's (FEMA) GRAS list. However, additives may form toxic pyrolysis products if used in tobacco products for smoking. The fact that an additive has GRAS status does not necessarily mean that it is "safe" in tobacco products.

The authors concluded from the data obtained in studies, which had examined additives at realistic (and not experimentally inflated) inclusion levels, that no relevant increase of the biological activity of mainstream smoke (cytotoxicity, mutagenicity or carcinogenicity) was shown for cigarettes, which contained the additives.

- RODGMAN (21, 225, 226)

RODGMAN reviewed the effects of additives on cigarette mainstream smoke properties in three consecutive papers dealing with flavorants (225), casing materials and humectants (21), and ingredients reportedly used in various commercial cigarette products in the U.S.A. and elsewhere (226). The three papers derive substantive benefit from the author's personal involvement from the early 1950s to the late 1980s in a major company's tobacco and tobacco smoke composition studies and his continual collecting of the pertinent published literature from the early 1950s on.

The persistent theme of the first paper on flavorants (225) is the potential formation of hazardous mainstream smoke constituents from flavor additives compared to natural tobacco components. Almost all flavorants have a relatively low molecular weight (generally less than 200) resulting in high volatility under the conditions of smoking and enabling them to escape rather quickly and largely intact from the burning or heated tobacco rod. Many natural components of tobacco, however, have relatively high molecular weights (many are polymers) with low or no volatility. Consequently, they are subject to decomposition and pyrolysis and, as precursors, may give rise to harmful compounds, such as polycyclic aromatic hydrocarbons. Reviewing earlier research (conducted from the 1960s until about 1990), which examined flavorants (and a few other components) individually for their effect on cigarette mainstream smoke composition, RODGMAN compiled some thirty examples with focus on the efficiency of transfer into mainstream smoke. Other quoted studies done in the 1950s, 1960s and 1980s indicated that certain components in flavor formulations, which were assumed to generate undesirable components during smoking, did not. Interestingly, an early (1977) comparative study of the mutagenicity (Ames assay) of mainstream smoke condensates was done with cigarettes made from five different commercial blends, which contained either their brand-specific flavorant and casing/humectants formulations (equivalent to the marketed products) or ten times the flavorant formulation without casing/humectants or the usual casing/humectants formulation without any flavorants or no additives at all (control cigarettes). This way, among the five brands studied, a total of more than 150 different additives was captured. For all blends and combinations of additives, no substantive increase in mutagenicity was observed compared to control cigarettes (227). In conclusion, RODGMAN joined the assessment of DOULL *et al.* (223) and PASCHKE *et al.* (224) "*that the components of the flavorant formulation ("top dressing") added to tobacco in the manufacture of cigarettes are not hazardous under the conditions of use*".

In the second paper (21), RODGMAN reviewed certain aspects of the use of casing materials and humectants in cigarettes. A large number of internal tobacco industry documents (mainly from R.J. Reynolds), which have now become available on the internet (228), were evaluated to show an impressive amount of early research done on these additives from the late 1950s on. Because of their relatively high usage level compared to flavorants (except menthol) it was much easier to assess the effects of the casing materials, sugars, licorice and cocoa, on cigarette mainstream smoke, specifically the smoke levels of polycyclic aromatic hydrocarbons, phenols and aldehydes. Pyrolysis studies with mono-, di- and polysaccharides started as early as 1957 and continued heavily into the 1970s and 1980s. For licorice and cocoa, it was demonstrated that their use in cigarettes augmented many desirable flavor compounds in tobacco smoke, such as pyrazines. The concern that glycyrrhizic acid, a major component of licorice, may be a precursor of polycyclic aromatic hydrocarbons, particularly B[*a*]P, was shown to be unwarranted.

Studies that were reviewed on the analysis, fate and biology of humectants (glycerol, 1,2-propylene glycol and the rarely used triethylene glycol) in tobacco and tobacco smoke began in 1957. One of the topics was the potential conversion of glycerol to acrolein. The quantitative contribution of humectants (by simple transfer) to cigarette smoke condensate is particularly interesting because of the reduction by dilution of analyte concentrations and biological effects when high amounts of these additives are used. The comparative mutagenicity study with cigarettes made from five different commercial blends with and without their brand-specific casing/humectants formulations (227) was already mentioned above. The first commissioned mutagenicity study with neat humectants dates back to 1979, followed by a number of *in vitro* and inhalation studies in 1987–1990.

RODGMAN concluded that the casing materials (sugars, cocoa and licorice) and humectants (glycerol and 1,2-propylene glycol) added to tobacco during cigarette manufacturing were not hazardous under the conditions of use.

The new and useful parts of RODGMAN'S third paper (226) are several diligently compiled tables with a wealth of references to new research published until 2004, or earlier research now available on the internet (228). The starting point for the tables was DOULL'S list (223), released in 1994, of the 599 additives used by U.S. cigarette manufacturers. One table is focused on the 460 individual compounds on the DOULL list (with their CAS numbers and Chemical Abstracts names) providing literature references of studies on (a) their pyrolysis products, (b) their effect on the chemical and biological properties (*in vitro* mutagenicity and genotoxicity, and *in vivo* tumorigenicity assayed in inhalation and skin painting studies) of cigarette mainstream smoke, and (c) their demonstration in untreated cigarette tobacco and/or its mainstream smoke. A companion table looks at the mixtures on the DOULL list, such as oils, resins or plant extracts, identifying studies on their effect on the chemical and biological properties of cigarette mainstream smoke. Reaching out beyond the DOULL list, RODGMAN identified additional additives used outside the U.S. For the 50 individual compounds and 34 mixtures, tables were prepared in formats identical to the ones for the materials on the DOULL list.

Interesting information was compiled in another table showing, which individual flavor compounds (found on the DOULL list) were identified in seven flavor mixtures (on the DOULL list) and the two casing materials, cocoa and licorice (both also on the DOULL list). A further table contains a listing of studies on tobacco additives conducted from the mid 1990s up to the year 2003 and reproduces their main findings and conclusions.

4.4. Comprehensive experimental studies

Mainly in response to (anticipated) regulatory requirements, the major cigarette manufacturers have in recent years executed a number of large and highly ambitious research projects in order to evaluate the toxicity of tobacco additives used in cigarette production. The studies were focused on the effects of additives on mainstream smoke composition and activity in biological *in vitro* and *in vivo* test systems.

These effects of additives may be investigated in two ways. Mixtures of additives (in some studies comprising over 100 components) may be used with the intention of reproducing realistic (manufacturing) conditions as closely as possible and capturing possible interactions between the substances. The downside of this approach are quantitative limitations in putting the mixtures onto the tobacco (to avoid undesirable changes of the burning characteristics of the test pieces), which restrict dose-response testing, and the practical impossibility of relating specific effects to individual additives. Alternatively, additives may be examined as single substances. This allows the direct assessment of additive effects in the appropriate matrix, tobacco. However, in view of the large number of substances that may be added to tobacco in cigarette manufacturing the task involves a high volume of testing and requires a considerable amount of time and effort.

- CARMINES (229), RUSTEMEIER *et al.* (230), ROEMER *et al.* (231) and VANSCHEEUWIJCK *et al.* (232)

In 2002, CARMINES (229) described a testing program designed to evaluate the potential effects of 333 commonly used additives - as mixtures - on selected chemical and biological endpoints in cigarette mainstream smoke. The experimental cigarettes were made of a typical commercial U.S. tobacco blend. For testing, the additives were divided into three groups. The first group included casing materials, volatile top flavors and substances found in reconstituted tobacco sheet; the second group comprised casing materials and volatile top flavors; and the third group consisted of selected high-load casing materials (cocoa shells, licorice extract and corn syrup) and *l*-menthol. The groups were sorted together in line with normal application practices to allow the evaluation of potential synergistic effects. But then, the potential effects of individual additives are difficult to recognize in this kind of study.

Three pairs of filter ventilated cigarettes (tip ventilation 30%) were produced, each containing one of three different groups of additives. In each pair, there were cigarettes with the usual use level of additives and others with a 1.5- to 3-fold level compared to normal use (called low-level and high-level cigarettes). The application of the additives under the condition of maintained tobacco filler weight did not significantly alter the burning characteristics of the test cigarettes (as evidenced by very comparable puff numbers) but produced elevated TPM levels (by 13–28%). Between 7% and 15% of the tobacco was replaced by the additive mixtures in the six different experimental cigarettes. The purpose of the study was the determination - and publication in separate papers - of the effects of additives on 51 selected mainstream smoke constituents (230), on *in vitro* toxicity, assessed in the Ames *Salmonella* reverse mutagenicity test and the mouse embryo cell cytotoxicity (neutral red uptake) assay (231), and in 90-day nose-only smoke inhalation studies in rats (232).

The results of mainstream smoke analysis were presented comprehensively in a table on a per cigarette basis. Following normalization by TPM, radar charts were prepared showing smoke constituent amouts for each of the three additive groups (both inclusion levels) in test cigarettes relative to control cigarettes. Overall, smoke chemistry data indicated a reduction of the majority of smoke constituents and a few increases when normalized to TPM yields and compared to control cigarettes. These changes in mainstream smoke composition, while statistically significant, were not reflected by any significant alterations in the biological activity of cigarette smoke as shown in the Ames tests, cytotoxicity assays and subchronic inhalation studies in rats. Based on the results of these examinations CARMINES (229) concluded that the substances added to tobacco "*do not add significantly to the overall toxicity of cigarettes*". Details and the outcome of these tests are discussed in the respective chapters of this review.

In a recent paper of suggestive nature the work of CARMINES and colleagues (229–232) was criticized by WERTZ *et al.* (233). The authors combined the analysis, recalculation and interpretation of the scientific data with speculations and incriminations based on the picking and squeezing of "tobacco industry documents" in the Legacy To-

bacco Documents Library. WERTZ *et al.* recapped the analytical data of CARMINES and colleagues in radar charts on a per cigarette basis - with data taken straight from the original paper (230) - and additionally after adjustment by smoke nicotine. While data readjustment is a scientifically acceptable procedure they went further claiming that the adjustment of data by TPM, as done by CARMINES and colleagues, was a post-hoc change of the analytical protocols after initial statistical findings indicated an additive-associated increase in cigarette toxicity that needed to be concealed. However, adjustment by TPM was the conclusion of a complex internal discussion by the scientists involved - a fact WERTZ *et al.* failed to recognize from the internet documents preyed upon. In the judgment of CARMINES and colleagues, TPM was the reference parameter for smoke data that allowed the most realistic consideration of the several technical variables influencing smoke yields (cigarette density, amount of burned tobacco, etc.). In addition, WERTZ *et al.* accused CARMINES and colleagues of the inaccurate execution of subchronic smoke inhalation studies, essentially because of insufficient number of animals and exposure time. To demonstrate that more powerful experimental conditions would have revealed increased additive related toxicity they enlarged fictitiously the number of animals more than fivefold and, at the same time, took the descriptive statistics (means and standard deviations) published by CARMINES and colleagues unchanged for a mock recalculation. Not surprisingly, the recalculation produced the desired (but frivolous) result.

DEMPSEY (234) of Philip Morris International responded to the critique by pointing out that the arguments and recalculations of WERTZ *et al.* (233) did in no way invalidate the conclusion arrived at by CARMINES (229) that cigarettes with additives were not more toxic than additive free cigarettes. In their reply WERTZ *et al.* (234) repeated the critique without providing new or convincing arguments and did not comment on any of DEMPSEY'S specific statements. Another reaction to the paper of WERTZ *et al.* (233) came from OLDHAM and MCKINNEY of Altria Client Services (235). They pointed out that the approach and methods used (229–232) were based on sound toxicological principles and guidelines. The testing program was developed by adapting ways of toxicological evaluation by the U.S. FOOD AND DRUG ADMINISTRATION for food additives (236) and proposed by the CONSUMER PRODUCT SAFETY COMMISSION for cigarettes (237). Classical toxicological evaluation assays and internationally recognized methods were adapted for use with cigarette mainstream smoke. OLDHAM and MCKINNEY also pointed out that the purpose of this program was to ensure that additives used in cigarettes do not increase the inherent toxicity of cigarette smoke. The results and conclusions were consistent with those from other cigarette additive studies and with human epidemiological evidence indicating that smoke from cigarettes with additives was no more hazardous for health than smoke from additive free cigarettes (238). In their reply, WERTZ *et al.* (235) stated that OLDHAM and MCKINNEY sidestepped the most important points of critique of the studies by CARMINES and colleagues (229–232), and repeated their arguments without presenting new facts.

- BAKER *et al.* (207, 208, 239)

In 2004, BAKER *et al.* (207, 208, 239) reported on a full-scale research program with the objective of assessing the effects of a large number of additives on the composition and toxicity of cigarette mainstream smoke. This course of action is to be seen in close connection with BAKER and BISHOP'S concurrent pyrolysis studies (199, 200), which are discussed above (see Section 4.1. on page 425).

Three series of test pieces (A, B and C) were produced together with their respective control cigarettes. For laying out the study "*as authentic as possible*" the ready-made tobacco portion in series A (examining flavor additives) consisted of a blend of lamina, expanded tobacco, cut rolled stems and reconstituted tobacco sheet (comparable to a typical U.S. tobacco blend), which was then treated with a standard casing mixture (and subsequently loaded with the additives under investigation); in series B (checking three combinations of casing materials and flavors as well as additives contained in an experimental reconstituted sheet) of lamina, expanded tobacco and cut rolled stems; and in series C (checking several casing materials and humectants) of lamina and reconstituted tobacco sheet. The purpose of using varied combinations of flavor and casings additives was the detection of possible interactions between different components.

All manufactured cigarettes (with no tip ventilation) were designed to produce an ISO "tar" yield of about 13 mg. This level was chosen to ensure that all "Hoffmann analytes" in smoke would be above their detection limits, and to maximize any effects of the additives. Reference cigarettes (the Kentucky 1R4F and two internal standards) were included in the smoking runs.

A total of 482 additives was added at their typical maximum use levels in various combinations, which were basically specified by experimental convenience, resulting in 19 different test pieces in series A, B and C. Interestingly, in the B series one test piece examined a simple (three-component) casing mixture in combination with menthol as the only flavor (2.34% on the tobacco), another test piece looked into an experimental sheet (8.8% in the ready-made tobacco portion) with typical components (flavors and binders, calcium carbonate and glycerol) but no other flavor or casing materials present; and in the C series one test piece inspected only water plus propylene glycol, the two "carriers" used for casing mixtures. Altogether, the additives comprised 462 flavors, 1 flavor/solvent (triacetin), 1 solvent (ethanol), 7 preservatives, 5 binders, 3 humectants, 1 filler (calcium carbonate) and 2 processing aids (ascorbic acid and water).

For cigarettes of the A series, the inclusion levels of the seven flavor mixtures, each containing between 14 and 73 different compounds, were 1.2–3.7 mg per test piece. Each flavor component was dosed in the study at a minimum of 12 ppm even though inclusion in commercial products may be as low as 0.5 ppm. In the B series, three different casing mixtures (two with only three and the other with 15 components, up to 75 mg per cigarette) were combined with two different flavor preparations (63–85 components at a total of about 5 mg per cigarette) or menthol. The B series test piece with experimental sheet was already mentioned. Seven mixtures of casing materials (2–16 components)

were applied in the C series at levels of 51–68 mg per cigarette (excluding water and propylene glycol), and the amount of the ready-made tobacco portion was reduced accordingly. As mentioned above, one test piece had only water and propylene glycol added.

The analysis of mainstream smoke composition was focused on the so-called "Hoffmann analytes", a compilation of 44 substances believed by regulatory authorities in the United States (204) and Canada (205) to have impact on smoking related diseases. Any statistically significant increases were of particular concern.

Regarding the A series, the addition of the seven different flavor mixtures had only occasional significant effects on analyte yield compared to control. Across all measurements and test cigarettes there were 14 significant increases (4.5% of all measurements), of which none were striking. For instance, NFDPM (nicotine free dry particulate matter = "tar") was elevated in two test pieces by 5.5% and 8.6%, resp.

In the B series with the three combined casing/flavor mixtures, the additives generally increased the yields of NFDPM (by up to 9%) and CO (by up to 7%) relative to control. The rise in NFDPM was assumed to be due to the high transfer rate of additives to smoke, while elevated CO was probably due to the thermal decomposition of added carbohydrates and other polymers. With the three flavor/casing mixtures, increases were also observed for some test cigarettes in other smoke constituent levels, such as ammonia, HCN and formaldehyde (up to 24%), the latter resulting from sugar pyrolysis. Remarkable decreases in smoke levels were observed with some additive mixtures for most of the tobacco specific nitrosamines (up to 24%), NO_x, most of the phenols (up to 34%), benzo[*a*]pyrene, and some of the aromatic amines and other organic compounds. In part, these reductions were attributed to the "dilution" effect when up to 14% of the cigarette tobacco was replaced by additives.

With the test piece in the B series, which contained the additives under investigation in form of an experimental sheet, the yields of five smoke carbonyls were elevated (in case of formaldehyde by 68%, the largest single increase seen in the whole study). The pyrolysis of cellulose and other polysaccharide materials (major components of the sheet) was thought to be responsible. On the other hand, all tobacco specific nitrosamines, phenols, nitrogenous bases and NO_x were reduced by up to 22%.

An interesting pattern appeared after the analysis of the C series, which examined seven mixtures of casing materials and the "carrier" system, propylene glycol and water. Of the 46 cases of significantly increased "Hoffmann analytes" in smoke (13.1% of the 352 measurements across all measurements and test cigarettes), two thirds (31 cases) involved carbonyl compounds, notably formaldehyde, acetaldehyde, propionaldehyde and acrolein. While most increases did not exceed 15%, they were higher with four casing mixtures for formaldehyde and with two mixtures for acrolein. Of the remaining 15 increases of other (non-carbonyl) "Hoffmann analytes", only three were somewhat above 15%.

Taking a look at some of the specific casing mixtures it is worth noting that the mixture of propylene glycol (applied over eight times higher than in other mixtures) and water produced only a small increase of propionaldehyde, that the mixture containing little acetic acid and a lot of white sugar showed only increases in aldehydes (two quite moderate and the large one in formaldehyde), and that with the mixture of high-fructose corn syrup and cocoa powder ten of the 44 "Hoffmann analytes" were significantly increased (six carbonyls, 1- and 2-aminobiphenyl, and 1- and 2-aminonaphthalene).

The specific biological activity of the total particulate matter of test cigarettes compared to control cigarettes was determined in three *in vitro* bioassays for genotoxicity and cytotoxicity (Ames test, neutral red uptake and mammalian cell micronucleus assay) (239). Considering the sensitivity and specificity of these bioassays, the specific activity of cigarette mainstream smoke total particulate matter was not changed by the use of additives.

In 90-day sub-chronic inhalation studies in rats the response in the respiratory tract to mainstream smoke was not distinguishable between test and control cigarettes, neither in histomorphometric nor in histopathological assessments. The additives tested produced no discernible differences in the type and severity of treatment-related changes between the smoke generated from additive containing and additive free cigarettes. In summary, no significant influence of additives on cigarette mainstream smoke toxicology was detected (239).

Moreover, two points of special interest regarding the use of additives were highlighted by the extensive analytical work by BAKER *et al.* (207). It is to be expected that added volatile materials may be lost from the tobacco during manufacturing and/or storage. For certain solvents/carriers the losses were found to be quite remarkable after six months: ethanol had decreased by over 99%, propylene glycol by 55–65%, glycerol by 20–29% and triacetin by 54–71% (with about 20% recovered on the filter).

The comparison of different experimental cigarettes showed no relationship between ammonium levels in the blend (either increased by the addition of an ammonium compound or decreased by "dilution" with other additives), ammonia smoke yields and "smoke pH". This finding obviously contradicts speculations about ammonium additives and nicotine uptake by the smoker (17).

- RENNE *et al.* (240)

RENNE *et al.* (240) studied the effects of flavoring and casing additives on the *in vitro* and *in vivo* toxicity of cigarette mainstream smoke. In two studies, test cigarettes were compared to control cigarettes without additives. In the first study, the tobacco blend contained a mixture of 165 low-use additives, primarily found in top flavor formulations; in the second study, the tobacco blend was loaded with a mixture of eight high-use flavoring or casing materials: invert sugar, block chocolate, plum extract, fig extract, molasse extract and tincture, gentiana root extract, lovage extract and peppermint oil. Application rates exceeded normal use levels (listed in tables for each additive). The Ames *Salmonella typhimurium* assays (five strains with and without metabolic activation by the S-9 mix) did not show any increase in the mutagenicity of mainstream smoke condensates for the additive containing cigarettes compared to control cigarettes. Groups of

Sprague-Dawley rats were exposed to nose-only inhalation for 1 hour/day and 5 days/week for 13 weeks to three concentrations of mainstream smoke (0.06, 0.2 and 0.8 mg TPM/liter air) of the test and control cigarettes. No toxicologically meaningful differences were detected in biomarkers (carboxyhemoglobin, plasma nicotine), clinical pathology or the histology of the respiratory tract at any concentration tested. The authors concluded that the results obtained did not show any consistent differences in toxicological effects between the smoke from cigarettes containing flavoring or casing additives and the additive free control cigarettes.

- GAWORSKI *et al.* and COGGINS *et al.* (241–251)

In 2011, GAWORSKI *et al.* (241) outlined the rationale, approach and methodology of a comprehensive evaluation of the toxicity of 95 additives used in cigarette manufacturing. Each substance was examined individually and generally added at three different inclusion levels to the tobacco of experimental cigarettes for comparison to matched control cigarettes that were constructed concurrently and contained no additive. Kentucky reference cigarettes were included in the studies.
The additives examined individually were categorized in nine groups with a separate follow-up publication dedicated to each group: Various natural sugars and carbohydrate materials, including sorbitol, sucrose, invert sugar, high fructose corn syrup and honey - 11 compounds (242), a broad range of essential oils, plant and fruit extracts, gums and resins - 32 compounds (243), cocoa-derived products - 5 compounds (244), heterocyclic nitrogen compounds - 3 compounds (245), aromatic carbonyls - 10 compounds, namely raspberry ketone (4-(4-hydroxyphenyl) butan-2-one), acetophenone, benzaldehyde, cinnamaldehyde, ethyl vanillin, *p*-anisaldehyde, methyl phenylacetate, heliotropin, floranol and phenethyl phenylacetate (246), aliphatic carbonyls - 15 compounds (247), aliphatic and aromatic carboxylic acids - 9 compounds (248), aliphatic and aromatic alcohols - 8 compounds (249), and the two inorganic compounds, diammonium hydrogen phosphate and ammonium hydroxide (250).
The additives were tested individually using different amounts added to experimental cigarettes. Machine smoking of the cigarettes was done according to ISO (73). Mainstream smoke chemistry analysis for up to 52 analytes, and cytotoxicity (neutral red uptake) and bacterial mutagenicity (Ames) tests were conducted with all 95 additives. In addition, 31 of the additives were singled out, based in their customary addition to cigarettes at concentrations ≥ 100 ppm, for assessing the *in vivo* toxicity of mainstream smoke in 90-day nose-only inhalation studies in Sprague-Dawley rats.
Summarizing the wealth of data produced over a period of seven years by analyzing the mainstream smoke constituents of experimental and control (and reference) cigarettes it is worth noting that 13 of the 52 analytes measured were consistently below the limit of quantification and that the yields of another 24 analytes were never more than 25% increased, or even reduced, with any of the experimental cigarettes compared to control cigarettes. Some of the individual additives, when applied at very high levels, had a more pronounced effect on the yields of certain analytes. This, however, was not reflected in the biological endpoints of the *in vitro* and *in vivo* toxicity tests (cytotoxicity, mutagenicity and inhalation study data) where only minimal changes in the overall toxicity profile of test cigarette mainstream smoke were observed. Occasional small reductions of biological toxicity, noticed in cases of high additive inclusion levels, were thought to be due to the replacement of burning tobacco by the additive.
After finishing this large research project, with data collected during a multi-year test program with a variety of tobacco additives from several chemical classes and at different addition levels, GAWORSKI *et al.* (251) stated: "*The results of our evaluation add to a growing body of the literature regarding a weight-of-evidence assessment of cigarette ingredient toxicity. When assessed against the variability of assay methodology, natural agricultural change, and manufacturing control, the ingredients studied here demonstrated little relevant influence on the mainstream cigarette smoke toxicity endpoints measured*."

4.5. Single additives, their properties and effect on cigarette mainstream smoke composition and in vitro and in vivo toxicity

In this section, the influence of single additives on the components of cigarette mainstream smoke is discussed. For practical reasons, the scope of single additives includes mainly substances focused on by health authorities (2, 3, 15–18, 252), such as menthol, humectants, sugars, cocoa and licorice. In addition, certain additives used in higher amounts in cigarette manufacturing, such as citric acid and triacetin, are also considered. For studying the effects of a specific additive on cigarette mainstream smoke composition the additive is applied to cigarette tobacco as a single substance or in a mixture with other additives. The latter approach is much closer to reality because possible interactions between different additives during smoking are also captured. Such studies were performed primarily during the last 10 years.
Studying the effects of an additive on the composition of cigarette mainstream smoke by chemical analytical methods does not provide the full picture of which impact this additive may have on the toxicity of smoke. Additional evidence is generated when the toxicity of the mainstream smoke of cigarettes containing an additive is evaluated by *in vitro* and *in vivo* biological assays.
The biological activity of tobacco additives in cigarette smoke is always evaluated against the strong (possibly overwhelming) background of biological effects resulting from (burned) tobacco. Consequently, the principal challenge is to determine toxicologically relevant differences, if there are any, between test pieces with and without the additive(s) under investigation. This brings up the important problem of the discriminatory power of the chemical-analytical methods and the standard toxicity assays currently in use. The question was addressed in a recent paper by OLDHAM *et al.* (253), which brings into focus a statistical characteristic, the minimum detectable difference (MDD), for the critical evaluation of data. In spite of the usefulness of this approach, the authors do not fail to emphasize the need for weight-of-evidence analysis by

experienced researchers in the toxicological examination of tobacco additives.

In the various biological tests with different endpoints the three main tobacco types (Virginia, Burley and Oriental) have been shown to produce rather diverse - and sometimes opposite - results. As early as 1963, WYNDER and HOFFMANN (254) demonstrated in a mouse skin painting study that mainstream smoke condensate of cigarettes made from Virginia tobaccos was more tumorigenic than that of Burley cigarettes. MIZUSAKI *et al.* reported in 1977 that in the Ames assay with *Salmonella typhimurium* strains TA 1538 (255) and TA 98 (256) - both with rat liver microsomal fractions (S-9 mix) - the mainstream smoke condensate of Burley cigarettes showed higher mutagenic activity on a per mg condensate basis than that of Virginia cigarettes. Using the *Salmonella typhimurium* strain TA 98 with S-9 mix, GAIROLA (257) confirmed these results. In a study using the Ames mutagenicity assay with the *Salmonella* strains TA 98 and TA 100 with S-9 mix added, ROEMER *et al.* (258) reported results comparable to those of MIZUSAKI *et al.* (255) and GAIROLA (257).

However, in an examination of three genetic endpoints (frequency of gene conversions, reverse mutations and mitotic cross-over) in *Saccharomyces cerevisiae,* GAIROLA (257) found that fresh smoke from cigarettes made of Virginia tobacco had higher potency than Burley cigarettes. In 2006, SCHRAMKE *et al.* (259) compared the mutagenic activity of mainstream smoke condensate of cigarettes made from Virginia, Oriental and Burley tobacco. Machine smoking was done following the FTC protocol (142). Besides the Ames mutagenicity assay the mouse lymphoma thymidine kinase assay (MLA) was used. The experimental procedure followed the microtiter plate version of the MLA according to COLE *et al.* (260). In this test, the specific mutagenic activity - with and without S-9 mix - of the mainstream smoke condensate of Burley cigarettes was statistically significantly lower, by up to 40%, than of Virginia and Oriental cigarettes. Cigarette smoke condensate was also examined in the Ames assay with the *Salmonella typhimurium* strains TA 98 and TA 100 in the presence of S-9. When the data of the Ames test were compared to the MLA in the presence of S-9, an inverse ranking of the specific mutagenic activity was observed. In the MLA, mainstream smoke condensate from the Oriental cigarettes had the highest mutagenic activity, followed by the Virginia and Burley smoke condensates. In the Ames assay, Burley smoke condensate had the highest mutagenic activity with both strains, followed by Virginia and Oriental smoke condensate.

It is interesting to note that - quite contrary to the well-established Ames assay - the ranking of the mutagenic activity of cigarette smoke determined with the MLA corresponds to the ranking of the carcinogenic activity in the mouse skin painting assay (254) and the convertogenicity in the yeast test system (257). SCHRAMKE *et al.* (259) pointed out that test systems with different biological endpoints may respond non-uniformly to distinct chemical classes or constituents in cigarette smoke and called for the complementary use of both assays, Ames and MLA, in evaluating mutagenic activity.

In the following, an overview is presented on the composition and *in vitro* and *in vivo* toxicity of the mainstream smoke of cigarettes containing additives discussed in the previous section. A number of other additives used in tobacco products as preservatives or flavorings were also subjected to toxicological evaluations. These include potassium sorbate (261), vanillin (262), ten aromatic carbonyl compounds (246) and the pyrazines, 2,3-diethylpyrazine and 2,3,5,6-tetramethylpyrazine, and 2-acetylpyridine (245). The studies revealed little or no relevant change in the overall toxicity profile of the smoke of cigarettes containing these substances compared to additive free cigarettes.

4.5.1. Menthol

- Use and toxicological assessment

Menthol is a monocyclic terpene alcohol with three asymmetric carbon atoms in the cyclohexane ring, giving rise to four pairs of optical isomers. The *l*(−)-menthol isomer exhibits the characteristic balanced peppermint odor and flavor and exerts a cooling effect when applied to skin (263).

Menthol has been classified as Generally Recognized as Safe (GRAS) for use in foods by the Flavor and Extract Manufacturers Association (FEMA) (264, 265). It is understood that the regulatory approval of menthol use in foods and other consumer products was not intended to address its use in tobacco products and cannot be relied on solely as a basis for judging menthol safety when used as an ingredient in smoking products. Even so, menthol is today explicitly approved for use as a flavoring ingredient in tobacco products in a number of countries with pertinent regulations, e.g., in Germany (266).

The pharmacological effects of neat *l*-menthol on the respiratory system and the skin as well as its toxicology were reviewed in 1994 by ECCLES (263). The FEMA GRAS assessment of 1996 (265) discussed and evaluated the topical, respiratory and systemic toxicity of *l*-menthol used as food flavor. In 2010, information on the toxicity of menthol employed as a cigarette flavoring agent was reviewed by HECK (267). Major points of this paper are summarized below.

Ames *Salmonella* mutagenicity testing (involving strains TA 92, TA 94, TA 98, TA 100, TA 1535 and TA 1537) with *d,l*-menthol both in the presence and absence of an S-9 mixture for metabolic activation was reported to be negative (268, 269). In a chromosome aberration test with a Chinese hamster fibroblast cell line the response was also negative (269). The chromosome aberration and sister chromatid exchange assays with Chinese hamster ovary cells showed no effects for *d,l*-menthol (270).

It was concluded from these studies that menthol did not represent a mutagenic or genotoxic hazard (267).

l-Menthol (synthetic or natural), practically the only form used as tobacco additive, was reported to provoke no skin sensitization in a guinea pig model (271). Occupational exposures to menthol vapors up to 39.4 mg/m³ air in a working environment were noted to lead to slight respiratory and ocular irritation (272).

Menthol administered intraperitoneally to mice in a sub-chronic carcinogenicity study showed no effects (273). Equally, when *d,l*-mentol was given orally to rats and mice

in a chronic study (274) no carcinogenic activity were observed. A study on cancer chemoprevention (275) demonstrated protection by menthol in the diet against induced neoplasia in rats.
As shown in animals, the inhalation of menthol containing vapors had no adverse effects on mucociliary and phagocytic clearance (276). RIECHELMANN *et al.* (277) found a dose dependent decrease of ciliary beat activity in an *in vitro* study when freshly collected human nasal cells were exposed to mixed vapors of menthol, eucalyptus oil and pine needle oil. In our opinion, this effect cannot be unambiguously assigned to menthol in the vapor mixture tested.
RAKIETEN *et al.* (278) reported a subchronic study with rats inhaling pure *l*-menthol vapor at concentrations up to 0.259 ppm for 6.75 hours per day for 38 days. No obvious lethality was observed but severe pulmonary congestion and pneumonitis occurred at the highest exposure level.
In studies with pregnant CD-1 mice, Wistar rats, Golden hamsters and rabbits menthol showed no potential for adverse effects on development as reported in 2008 by the RIFM EXPERT PANEL (279).
Summarizing the studies mentioned above, menthol is not expected to show adverse effects on human health when used in doses, which correspond to mainstream smoke levels of 0.4–0.8 mg menthol per (U.S.) cigarette (280).

- Inclusion level in cigarettes, transfer and pyrolysis

Primarily *l*-menthol is used as tobacco additive, especially in manufacturing mentholated cigarettes. According to HOPP (271), a slight menthol effect is apparent at inclusion levels of 0.1–0.2% on tobacco and a stronger flavor note is achieved at 0.25–0.45%. As noted by HECK (267) some U.S. mentholated cigarette brands contain up to 2% menthol. The report of ALTRIA CLIENT SERVICES (280) includes an overview on the 2008–2009 U.S. menthol cigarette market. It was stated that menthol in cigarettes amounted to 0.33–1.39% relative to tobacco weight, the menthol content of mainstream smoke was 0.40–0.84 mg/cig (not considering cigarettes that deliver menthol from a capsule in the filter) and menthol transfer efficiency into mainstream smoke reached 7–21%, depending on cigarette construction.
The first data on the transfer of menthol into cigarette mainstream smoke were published in 1963 by MITCHELL *et al.* (281). About 20% of the menthol in the cigarette was found in the mainstream smoke of filter cigarettes. Using randomly labeled ^{14}C-menthol, NEWELL *et al.* (282) reported in 1968 its fate in burning filter cigarettes. Approximately 70% of the added menthol was found in the particulate matter of mainstream and sidestream smoke, much of the rest in the butt and filter. As much as 96.4% of the radioactivity found in mainstream smoke particulate matter represented intact menthol; this was also true for 91.7% of the activity recovered in sidestream smoke.
In 1970, the results of NEWELL *et al.* (282) were confirmed by JENKINS *et al.* (283). In their study they also used ^{14}C menthol. 28.9% of the radioactivity was found in mainstream smoke while 44.3% was detected in sidestream smoke and 26.9% in the butt. Of the cigarette mainstream smoke activity, 98.9% accounted for intact menthol, 0.1% for carbon dioxide, and the rest for other pyrolysis products. Mainstream smoke transfer efficiency was found to be about 10% of added menthol, depending on the construction of the cigarettes.
The mutual exchange of menthol between tobacco rod and cellulose acetate filter during storage as well as the apportionment of menthol during smoking to mainstream and sidestream smoke and the butt were investigated by BROZINSKI *et al.* in 1972 (284). The transfer of 20–25% of menthol into mainstream smoke was measured, and a considerable selective retention of menthol by cellulose acetate filters could be demonstrated.
Reviews of earlier studies on the transfer of menthol into mainstream smoke and the migration of the substance between cigarettes and packaging materials were presented by WILSON (63) and BEST (285) in 1993 at the Tobacco Chemists' Research Conference.
The influence of cigarette design on menthol transfer into mainstream smoke was studied by COOK *et al.* (286) using commercial cigarettes sampled from the U.S. market in the early 1990s. Menthol transfer into the mainstream smoke of filtered cigarettes of various designs and different menthol levels on tobacco ranged from about 3% for highly tip ventilated cigarettes (70% ventilation and more) to about 18% for non-ventilated products.
Concerning the fate of menthol in a burning cigarette it is no surprise that the results of NEWELL *et al.* (282) and JENKINS *et al.* (283) are definitely not compatible with the data obtained in 1968 by SCHMELTZ and SCHLOTZHAUER (287) showing pronounced degradation of menthol and the formation of benzo[*a*]pyrene and phenols under drastic pyrolysis conditions. Neat *d,l*-menthol was pyrolyzed in a quartz tube under a stream of nitrogen at 600 °C and 860 °C. At 860 °C, only 16% of intact menthol was identified in the pyrolysate but - besides other products such as benzene, toluene and phenols - 400 ppm benzo[*a*]pyrene was found. However, in no way can the pyrolysis conditions chosen in this study be compared to the conditions in a cigarette during puffing. This early work is mentioned only because it is still today inconsiderately referred to in a blunt statement like "*menthol combustion produces carcinogenic compounds such as benzo[a]pyrenes*" (288) or the suggestive comment "*that burning menthol at the same temperature as tobacco can produce a carcinogen*" (289).
Data on the influence of menthol as a tobacco additive on the distribution of particle size in cigarette mainstream and sidestream smoke were collected in a number of investigations. In a study originally designed to examine the exposure of 40 non-smokers to sidestream smoke in an environmental chamber BRINKMAN *et al.* (290) used two commercially available cigarette brands of two leading U.S. manufacturers, one mentholated and the other non-mentholated but both of different size and design, and found that the ratio of small particles (0.3–0.5 µm diameter) to somewhat larger ones (0.5–1.0 µm diameter) was elevated in sidestream smoke generated from the menthol brand compared to the non-menthol brand. Biomarker data measured in the 40 non-smokers exposed to sidestream cigarette smoke under controlled conditions were published by BERNERT *et al.* (291). The responses were relatively uniform among the non-smokers. Specifically, no consis-

tent differences were found when comparing mentholated and non-mentholated cigarettes.

Subsequently, BRINKMAN *et al.* (292) investigated the effect of menthol on the size distribution of fine and ultrafine particles in cigarette mainstream smoke. Two fairly similar and commercially available cigarette brands (mentholated and non-mentholated) manufactured by two different U.S. cigarette companies were smoked - in cross-over mode - by 9 subjects (5 male, 4 female) in a laboratory, and their smoking topography was recorded electronically. Smoking topography records were used for controlling a smoking machine to duplicate the individual smoking habits of the volunteers. The particles in the "simulated inhaled breath" generated this way were fractionated by size real-time by means of an electrical low pressure impactor (ELPI) into 12 particle size ranges (0.007–4.0 µm). With the mentholated cigarette 28% more ultrafine particles (< 0.1 µm in size) were produced compared to the non-mentholated cigarette. It is worth considering that "*the mass of ultrafine particulate was three orders of magnitude smaller than that of fine particulate*" (293). It should be noted that of the nine participants only one was a regular and another was an occasional menthol smoker, and that the menthol and non-menthol test cigarettes caused significant differences in terms of smoking behavior.

Recently, this study (originally presented in 2009 as a poster) was published in an extended version by BRINKMAN *et al.* (293) with more detailed analytical data. Physical and chemical measurements on the two test cigarettes, subject characteristics and smoking topography data were provided. For assessing particle deposition and smoke constituent uptake, particulate matter was characterized in "simulated inhaled breath" and real exhaled breath, and nicotine and two carcinogens, NNK and B[*a*]P, were measured in "simulated inhaled breath", in real exhaled breath and - as mouth level exposure (MLE) - in butts collected after home smoking. Participant-specific uptake of NNK (simulated and daily MLE) was higher with the mentholated cigarette while nicotine was not significantly different. Also, participants retained more ultrafine particles, and fine particulate B[*a*]P when smoking the menthol cigarette. This, however, was not reflected by the relevant biomarkers in spot urine: NNAL and NNAL glucuronide, cotinine and 1-hydroxypyrene levels were not different.

The authors pointed out that their publication was the first report evaluating how menthol may affect mainstream smoke particle size distribution, composition and deposition. The results showed that the smoking behavior of the volunteers and the post-puff inspiration/expiration data were significantly different when mentholated cigarettes were compared to non-mentholated. A plausible explanation of the influence of menthol on the number and size distribution of cigarette mainstream smoke particles was not offered.

Particle size distribution was determined by BRINKMAN *et al.* (290, 292, 293) in both studies with an electrical low pressure impactor (ELPI). There is no information in their publications concerning dead volumes, ageing time of smoke in the equipment, degree of air dilution of the smoke or pressure conditions in the ELPI. Mainstream smoke flowed directly from the smoking machine outlet to the ELPI interface, consisting of a stainless steel chamber. Heated air (60 °C, 4% relative humidity) passed through the chamber to align particle load with the measuring system. As noted above, more than 96% of menthol is found in the particles of cigarette mainstream smoke. Due to the high volatility of menthol the observed smaller size of smoke particles generated from mentholated compared to non-mentholated cigarettes is to be expected, depending on the dilution of the smoke aerosol. The conditions in the interface may also contribute to a change in particle diameter. In our opinion, the higher number of ultrafine particles in the mainstream smoke of the mentholated cigarettes, observed by BRINKMAN *et al.* (290, 292, 293), may well result from the conditions in the analytical system and should, therefore, be confirmed.

The results and conclusions of BRINKMAN *et al.* (290, 292, 293) should be valued against the background of several earlier studies published on the size and size distribution of particles in cigarette smoke.

In 1978, HINDS (294) had reported that rapid growth of tobacco smoke particles by ageing was observed with time, reducing the number of particles. On the other hand, coagulation could be retarded by rapid dilution with air, causing a reduction of particle size because of increased evaporation of volatile compounds from the particles. The degree of reduction depended on smoke dilution.

INGEBRETHSEN (295) described a light scattering method for determining the particle size distribution of undiluted and minimally aged mainstream smoke, which allowed measurements on a sufficiently rapid time scale to reveal changes in particle size taking place during the puffing of a cigarette. Attention was also paid to the influence of increasing cigarette ventilation. A mean average diameter of cigarette mainstream smoke particles in 35 mL puffs of 0.22–0.27 µm was reported, independent from puff number and tip ventilation (up to 84%).

The effects of aging time and dilution on particle size distribution were examined by CHEN *et al.* (296). Their results suggested that there was a dilution value critical for the rapid coagulation and evaporation, and the final particle size of the cigarette smoke aerosol - with further dilution having little effect on the decrease of particle size.

In a summary of data available in 2003, BERNSTEIN (297) stated "*...that the particle size emitted is similar for all cigarettes studied, whether filtered, nonfiltered, ventilated, or ventilated with the ventilation holes blocked*". The cigarettes studied included only one menthol brand. Therefore, the confirmation of BERNSTEIN'S statement is required for mentholated cigarettes.

VAN DIJK *et al.* (298) investigated in real time the amount of nanoparticles (size range 6–50 nm) in fresh and undiluted mainstream smoke of six commercial cigarettes (with "tar" yields of 1–10 mg). Test cigarettes were smoked not according to ISO (73) but with 50 mL puffs of 2-sec duration at 30-sec intervals. Nanoparticles were detected abundantly over the whole size range but their share of mainstream smoke particles was only a few percent, decreasing for smaller particles. The production of nanoparticles seems to require minimal smoking intensity and is related to particle size, filter ventilation holes, butt length, and claimed "tar" values. Similar to most other studies, the

work of VAN DIJK *et al.* did not include mentholated cigarette samples.

- Attractiveness and addictiveness

Menthol has been used as a cigarette flavoring ingredient since the late 1920s (299). Because of its unique minty taste and aroma menthol enjoys an exceptional position among tobacco additives and is one of the most intensively studied cigarette ingredients.

About 26% of the cigarettes sold in the United States are branded as mentholated, with black smokers showing a strong preference for this kind of cigarettes. Other countries show similar or even higher rates of menthol cigarette consumption (Hong Kong: 26%; Philippines: 60%) while the market share is lower in countries such as Finland (18%) or Australia (9–10%) and only marginal in, for instance, Canada (4%), the United Kingdom (3.9%) or Germany (1.3%) - all data were collected in 1999/2001 (300). The purposeful addition of menthol makes these products different from the classical American blend cigarettes. The top dressing of "non-menthol" American blend cigarettes may contain traces of menthol, complementing smoke taste and aroma (271). However, the menthol level in these cigarettes is so low that smokers cannot detect the specific smell, taste and feeling associated with menthol.

LAWRENCE *et al.* (301) prepared a review on the sensory properties of mentholated cigarettes and the effects of menthol on smoking topography. Forty-five publications were evaluated. Mentholated and non-mentholated cigarettes were compared in particular with respect to puffs per cigarette, puff volume, frequency and duration. The authors commented that the reviewed studies did not provide a clear picture of how menthol affected the topography of cigarette smoking because many studies suffered from methodological limitations.

According to the U.S. National Survey on Drug Use and Health (252), menthol may mask the harshness of cigarette smoke and, therefore, make it easier for adolescents to start smoking. However, it must be kept in mind that "harshness" was imprecisely defined and assessed using subjective measures. It was speculated that menthol cigarette smoking may influence the development of tobacco addiction (302). The hypothesis that menthol in cigarette smoke facilitates the initiation of smoking and makes smoking cessation more difficult was promoted by CONNOLLY (187) and others. KRESLAKE *et al.* (303) insinuated on the basis of internal, non-scientific industry documents that the tobacco companies manipulated the sensory characteristics of cigarettes, including their menthol content, this way facilitating smoking initiation and enforcing nicotine dependence. The menthol brands used in this strategy were alleged to be very successful in attracting young smokers and increasing brand popularity. Convincing arguments for this accusation were not presented by KRESLAKE *et al.* (303). The statements and claims made in the paper of KRESLAKE *et al.* were heavily criticized by the U.S. CENTER FOR REGULATORY EFFECTIVENESS in a letter (304) mailed to the Center for Tobacco Products of the U.S. Food and Drug Administration and rejected as inaccurate and strongly biased.

Fortunately, a number of serious scientific studies were published on the relationship between menthol and the attractiveness and addictiveness of cigarettes.

In 2002, HYLAND *et al.* (305) explored possible associations between mentholated cigarette use and a variety of indicators of nicotine dependence. 13,268 individuals participated in this study. Menthol smokers were found not to be different from non-menthol smokers in several parameters: The number of cigarettes smoked daily, the time of day of smoking the first cigarette, the age when a smoking career started and the degree of success of subsequent smoking cessation attempts. No consistent association was observed between these indices of dependence and cigarette mentholation in overall or race specific comparisons. It was concluded in this rather large study that menthol in cigarette smoke did not have any meaningful effect on the behavioral indices of the nicotine dependence under investigation.

In a cross-sectional analysis of data from 19,545 ever smokers, MUSCAT *et al.* (306) found that both black and white smokers of mentholated cigarettes consumed fewer cigarettes per day than smokers of other cigarettes. The use of mentholated cigarettes was not related to quit rates in both blacks and whites. The authors concluded "*that menthol does not increase the addictive properties of tobacco nicotine*".

OKUYEMI *et al.* (307) observed no difference in addiction between menthol and non-menthol cigarette smokers in a cross-sectional survey of 480 Afro-american smokers. According to the authors, despite the limitations noted (limited sample size, self reporting without confirmation, risk of false reporting and recall bias) the data suggested that Afro-american menthol smokers were less successful with smoking cessation.

Three years later, in 2007, OKUYEMI *et al.* (308) presented an assessment of the relationship of mentholated cigarettes and smoking cessation among Afro-american light smokers (less than 10 cigarettes/day). The authors concluded from the data obtained in an earlier study (309, 310) with 755 young Afro-americans participating in a clinical trial on smoking cessation that "*among African American light smokers, use of menthol cigarettes is associated with lower smoking cessation rates*". The conclusion was criticized by the CENTER FOR REGULATORY EFFECTIVENESS for various shortcomings (311). The primary objective of the original study (309) had been to examine the efficacy of nicotine replacement therapy (nicotine chewing gum *vs.* nicotine free placebo) among Africo-american smokers consuming less than 10 cigarettes per day, and the data were afterwards converted to test the hypothesis that menthol smokers were less likely to quit than non-menthol smokers. The 755 subjects considered in the assessment included 615 menthol smokers and only 140 non-menthol smokers. While it was reasonable to expect a number of menthol smokers being unable to quit smoking, the roughly 4.5:1 ratio of menthol to non-menthol smokers probably resulted in an inflated/lop-sided comparison. There was a significant attrition rate of participants to show-up in the original study for their follow-up assessments. This means it was not possible to demonstrate the success of smoking cessation in all participants and, consequently, to verify the post-hoc hypothesis of a difference in smoking cessation between

users of mentholated and non-mentholated cigarettes. Therefore, the conclusions of OKUYEMI *et al.* must be regarded with considerable caution.

HÉBERT (289) raised the question whether menthol promoted the absorption of nicotine in the mouth and lungs during smoking because numerous studies had shown that *l*-menthol (the optical isomer naturally present in mint and primarily used in the mentholation of cigarettes) enhanced the dermal absorption of pharmaceutical agents (312).

The effect of menthol on nicotine pharmacokinetics in rats after cigarette smoke inhalation was studied recently by ABOBO *et al.* (313). Single mentholated cigarettes decreased the mean peak concentrations of nicotine in plasma significantly from 27.1 ng/mL to 9.61 ng/mL and the total area under the plasma concentration-time curves from 977 to 391 ng·min/mL compared to non-mentholated cigarettes of the same brand. After multiple smoke inhalation the peak concentrations of nicotine in plasma, the total areas under the plasma concentration-time curves, and the average steady-state plasma concentrations of nicotine and cotinine were also significantly lower in rats with mentholated cigarettes compared to non-mentholated. According to the authors these results suggested that menthol in cigarettes may substantially decrease the absorption, and/or increase the clearance, of nicotine in rats. ABOBO *et al.* discussed the extrapolation of their rat data to the smoking topography of human menthol smokers. In our opinion, their approach draws on unproven hypotheses, assumptions and speculations, and should be regarded with caution.

In 2007, WERLEY *et al.* (314) reviewed the possible effects of smoking mentholated cigarettes. They discussed the possibility of menthol promoting the absorption of nicotine in a smoker's mouth and lungs. Contrary to dermal absorption they did not find any published research showing increased menthol-mediated absorption of any xenobiotic chemicals in the oral cavity or lungs. On the basis of the different physiological conditions between the epidermis and the mucosa in the mouth and lungs, they concluded that there was no apparent reason to believe that mentholation affected the absorption of nicotine from inhaled cigarette smoke.

At the 2005 National Conference or Tobacco and Health (NCTH Meeting), Chicago, LI *et al.* (315) presented a cohort study comparing the nicotine dependence of menthol and non-menthol cigarette smokers. No consistent differences were found for the indicators of nicotine dependence between smokers of menthol and non-mentholated cigarettes.

FAGAN *et al.* (316) investigated nicotine dependence and quitting behavior among U.S. smokers of mentholated and non-mentholated cigarettes with similar consumption patterns. The 2003 and 2006/07 U.S. Tobacco Use Supplements to the U.S. Current Population Surveys (317) were pooled to conduct secondary data analysis. National data were collected using in-person and computer-assisted telephone interviews. Data from 46,273 current smokers aged 18 years and older were evaluated. Menthol smokers reported a mean consumption of 13.05 cigarettes per day compared with 15.01 cigarettes per day among non-menthol cigarette smokers. Multivariate results showed for smokers consuming 6–10 cigarettes per day that menthol smokers were significantly more likely than non-menthol smokers to consume their first cigarette within 5 minutes after waking up. This, however, was not observed in smokers consuming more than 10 cigarettes per day. The multivariate models did not show significant associations between usual cigarette brand and quitting attempts. Mean cigarettes smoked per day and the FAGERSTROM test for nicotine dependence (100) did not differ significantly for menthol and non-menthol smokers. The authors concluded from their findings that adult menthol smokers reporting to consume 6–10 cigarettes per day showed stronger signs of nicotine dependence than comparable smokers of non-mentholated cigarettes - a conclusion that could not be drawn for subjects smoking more than 10 cigarettes per day.

In our opinion, the data do not support the hypothesis that smokers of mentholated cigarettes experienced greater difficulties in quitting attempts. FAGAN *et al.* pointed out that their study had some limitations. Data for this retrospective study were self-reported; consequently, poor memory, bias or external influence could not be excluded. There was no information on the cigarette brands consumed, smoking topography and possible brand switching. The menthol and nicotine content of the cigarettes was unknown. All these facts must be considered in evaluating this study.

In 2011, DELNEVO *et al.* (318) reevaluated the data used by FAGAN *et al.* (316) looking at smoking cessation rates among smokers of mentholated cigarettes. They stated that FAGAN *et al.* were not successful in finding a difference in quitting attempts or smoking cessation rates between users of menthol and non-menthol cigarettes because their focus was not on successful smoking cessation but on quitting attempts among those, who continued to smoke. The reevaluation showed that - contrary to FAGAN *et al.* (316) - smoking mentholated cigarettes was solidly associated with decreased cessation level, and that the association was more pronounced among black and Puerto Rican smokers. In our view, the limitations observed for the study by FAGAN *et al.* (316) apply as well to the analysis of DELNEVO *et al.* (318).

In 2011, HOFFMAN and SIMMONS (319) presented a review concerning menthol cigarette smoking and nicotine dependence. The authors wished to clarify the effect, if any, of menthol in cigarettes on nicotine dependence in young and adult smokers. Thirty-five relevant papers were used for the review. According to HOFFMAN and SIMMONS the majority of indicators of nicotine dependence, including night awaking to smoke and the time of the first cigarette after waking up, suggested in general that menthol cigarette smokers were more heavily dependent on nicotine. Other indicators of nicotine dependence, including the number of cigarettes smoked per day and the FAGERSTROM test for nicotine dependence (100), failed to consistently differentiate menthol and non-menthol smokers. According to the authors, these indicators are not thought to be as robust as the time of the first cigarette, as suggested by BAKER *et al.* (101). Scientifically convincing proof for this statement is not available either from HOFFMAN and SIMMONS (319) or from BAKER *et al.* (101).

Using questionnaire data of the representative U.S. National Youth Tobacco Surveys of 2000 (320) and 2002 (321), HERSEY *et al.* (322) evaluated in 2006 whether mentholated cigarette brands were favored by young

people, who started smoking. It was concluded that mentholated cigarettes induced tobacco consumption because this kind of cigarettes - in the authors' opinion - produced less harsh smoke. Due to survey response inconsistencies a certain degree of uncertainty regarding cigarette preference (mentholated or non-mentholated) pared down the validity of the study. The study was criticized by the U.S. CENTER FOR REGULATORY EFFECTIVENESS (304) as unreliable and possibly biased.

In an additional paper, published in 2010, HERSEY *et al.* (323) reviewed prior research and analyzed the 2006 National Youth Tobacco Survey (324), using logistic regression to assess the relationship between smoking menthol cigarettes and needing a cigarette within one hour after smoking. They found that smoking menthol cigarettes was significantly associated with reduced time of needing a cigarette compared to smokers consuming non-mentholated brands. They concluded that mentholated cigarettes contributed to the appeal of youth smoking and to the addictive potential of smoking cigarettes among youth.

WACKOWSKI and DELNEVO (325) investigated the relationship between smoking mentholated cigarettes and subjective measures of nicotine dependence. 1,345 current cigarette smoking adolescents in grades 9–12, who participated in the representative 2004 U.S. National Youth Tobacco Survey (326), were examined. 46% of self-reported smokers expressed a preference for mentholated cigarettes. Therefore, WACKOWSKI and DELNEVO concluded from the answers of smokers of mentholated in comparison to non-mentholated cigarettes that mentholated products may be more addictive than regular cigarettes in young smokers. However, the authors commented that the study had limitations as the data and conclusions were based on self-reports, which may have been affected by under- and over-reporting. In addition, the authors noted that the 2004 U.S. National Youth Tobacco Survey (326) was not designed to test hypotheses related to the use of mentholated cigarettes and the dependence on smoking.

In our opinion, self-reporting of smoking habits by adolescents in grades 9–12 may be appreciably biased and the answers in the questionnaires influenced by others. Therefore, the conclusions drawn by HERSEY *et al.* (322) and by WACKOWSKI and DELNEVO (325) must be regarded with considerable caution.

In 2006, COLLINS and MOOLCHAN (327) investigated in telephone interviews the smoking urge among adolescent menthol and non-menthol smokers seeking cessation treatment. There was no significant difference in the number of cigarettes smoked per day but smokers of mentholated cigarettes smoked their first cigarette earlier in the day than consumers of non-mentholated products. According to the authors this suggested greater addictive potential of mentholated compared to non-mentholated cigarettes. As noted by the CENTER FOR REGULATORY EFFECTIVENESS (304) the conclusions of COLLINS and MOOLCHAN are rather fragile because 45% of menthol cigarette users tended to smoke their first cigarette within five minutes after waking up compared to 29% of non-menthol smokers. This kind of observation was based on survey respondents' subjective estimates and was not confirmed by objective tests.

MURRAY *et al.* (328) reported on 5,887 male and female adult smokers participating in a clinical trial on smoking cessation, which was part of the U.S. Lung Health Study (329–331). Contrary to expectations regarding nicotine dependence, users of menthol cigarettes had smoked fewer pack-years at baseline (counted between November 1986 and April 1989). After 14 years, no difference in cessation success was seen in menthol and non-menthol smokers. No indication was found that the mentholation of cigarettes was a factor that contributed to the well-known risks of smoking as there were no differences in the risk ratios for coronary heart disease, cardiovascular disease, lung cancer or any smoking related death. The authors concluded that their data provided "*no evidence that mentholation of cigarettes increases the hazards of smoking*".

The hypothesis that the consumption of mentholated cigarettes was associated with lower abstinence rates when pharmaceutical cessation aids, such as nicotine replacement therapy or bupropion, were used was examined by FU *et al.* in 2008 (332). The results of this study suggested that smoking menthol cigarettes did not complicate smoking cessation among older smokers during a quitting attempt aided by pharmacotherapy.

In 2009, MUSCAT *et al.* (333) concluded from data obtained in a community-based cross sectional study with 525 black and white volunteers that there was no significant association between nicotine dependence and the use of menthol cigarettes, measured by means of the FAGERSTROM index (100). However, an increased probability for smoking soon after waking up was observed in smokers of menthol cigarettes compared to non-menthol smokers. In addition, the results showed that menthol did not affect the physiological exposure to tobacco smoke constituents, including nicotine, but indicated that menthol might inhibit the detoxification of the potent lung carcinogen, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanol (NNAL).

In 2010, FOULDS *et al.* (334) reviewed the open literature on the use of menthol cigarettes and the effectiveness of quitting smoking. They concluded that there was growing evidence that certain subgroups of smokers found it harder quitting menthol *versus* non-menthol cigarettes. However, additional studies were called for with reliable measurements of the cigarette brands used, socioeconomic status and biomarkers of nicotine uptake. This request was a clear indication of the uncertainties and weaknesses of most of the studies reviewed by the authors.

HOFFMAN and MICELI (335) reviewed the relationship between the use of mentholated cigarettes and smoking cessation behavior. Summarizing 20 published articles some studies had found that menthol smokers had less success in quitting smoking, while others had failed to see a significant difference between menthol and non-menthol smokers. Some clinical trials evaluating the effect of different cessation treatments, e.g., nicotine replacement therapy, had first suggested that smokers of mentholated cigarettes had poorer outcomes but two secondary data analysis studies, using the same original data set, could not find any difference in success rates associated with the treatments. In addition, a possible interaction between menthol cigarette smoking and race/ethnicity was suggested, with worse outcomes for adult Afro-american and Hispanic/Latino smokers than for white menthol smokers. There was no consistent relationship between mentholated cigarette use and quitting success for white smokers.

In 2012, MUSCAT *et al.* (336) evaluated the hypothesis that users of mentholated cigarettes allowed less time to pass after awaking than non-menthol cigarette users before smoking their first cigarette. They examined whether any statistical association reflected increased dependence by measuring nicotine uptake as plasma cotinine. The community based study included 495 black and white daily smokers. The results showed a tendency of blacks smoking the first menthol cigarette within a shorter time after awaking. According to the authors their study showed that while menthol in cigarettes was associated with an indicator of nicotine dependence in blacks, menthol was not associated with biological uptake of nicotine in black and white smokers. The data indicated that there was evidence that menthol was associated with a behavioral measure of nicotine dependence in black adult daily smokers, and also showed that this association did not implicate menthol as a factor in nicotine uptake in active adult black und white smokers

In 2011, BLOT *et al.* (337) published the results of a prospective cohort study with 85,806 racially diverse adults concerning the lung cancer risk among smokers of menthol cigarettes. Smokers were classified by preference for menthol *vs.* non-menthol cigarettes. (For epidemiological results, see Section 5.3.2. on page 467). As part of the study, smoking habits and smoking cessation rates were evaluated. Both black and white menthol smokers were noted smoking fewer cigarettes per day than non-menthol smokers. Smoking cessation rates did not differ remarkably between menthol and non-menthol cigarette smokers during an average of 4.3 years of follow-up (odds ratio = 1.02; 95% CI = 0.89 to 1.16).

In 2010, ALTRIA CLIENT SERVICES (280) submitted to the FDA on behalf of Philip Morris U.S.A. background information concerning menthol use in cigarette manufacturing and menthol effects on the consumers of mentholated cigarettes. This was done in response to a request from the Tobacco Products Scientific Advisory Committee (TPSAC). Besides technical aspects of manufacturing mentholated cigarettes the effects of menthol on cigarette attractiveness and addictiveness, smoking prevalence in adolescents, smoking cessation and smoking associated health risks were reviewed and evaluated on the basis of the published scientific literature and so far unpublished results of Philip Morris research. The conclusions of ALTRIA CLIENT SERVICES concerning the effects of cigarette mentholation are presented in the respective chapters of this review. With regard to attractiveness and addictiveness ALTRIA CLIENT SERVICES concluded in their review that menthol cigarettes did not appear to play a strong role for smoking initiation in young people and menthol did not increase cigarette dependence above the level of menthol free cigarettes. The personal decision to quit smoking and do it successfully was not influenced by the menthol content of cigarettes.

In 2011, an additional document concerning menthol as a cigarette additive was submitted to the FDA by the non-voting industry representatives of TPSAC and other U.S. tobacco industry stakeholders (338). This document was also based on the evaluation of the scientific literature. The various effects of mentholated cigarettes on smoking behavior, smoking prevalence and health risks were evaluated. The conclusions drawn by the authors are presented in the relevant chapters of this review. Concerning attractiveness and addictiveness it was stated that the evidence on smoking topography was inadequate to support the notion that menthol cigarettes influenced smoking initiation or were smoked more intensely. Based on the published literature menthol addition to cigarettes had a meaningful impact neither on nicotine dependence nor on smoking cessation.

In spite of the uncertainties in scientific background and the partly contradictory results of the different studies as presented in this review, the TOBACCO PRODUCTS SCIENTIFIC ADVISORY COMMITTEE (TPSAC) concluded in their report submitted in July 2011 to the FDA (339, on page 216-217): "*The evidence is sufficient to conclude that a relationship is more likely than not that the availability of menthol cigarettes increases experimentation and regular smoking. ... The evidence is sufficient to conclude that a relationship is more likely than not that the availability of menthol cigarettes increases the likelihood of addiction and the degree of addiction in youth smokers. ... There is insufficient evidence to conclude that menthol cigarettes increase the likelihood of addiction and the severity of addiction in adults. ... The evidence is sufficient to conclude that a relationship is more likely than not that the availability of menthol cigarettes results in lower likelihood of smoking cessation success in African Americans, compared to smoking non-menthol cigarettes.*"

- Effect on cigarette mainstream smoke composition

KAISERMAN and RICKERT (340) evaluated analytical data for individual brands of mentholated and non-mentholated cigarettes. They reported that mentholated cigarettes showed no increased mainstream smoke delivery of benzo[*a*]pyrene. This finding demonstrates again how misleading it can be to apply results of forced pyrolysis studies with a neat compound (287) to the situation in a burning cigarette.

Unpublished studies of the R.J. Reynolds Tobacco Co. compared the mainstream smoke yields of experimental filter cigarettes containing 1.03% menthol on tobacco (6.68 mg/cig) to identical cigarettes without menthol (341). Besides "tar", smoke nicotine and the carbon oxides (CO and CO_2), menthol, ammonia, benzo[*a*]pyrene, formaldehyde, acetaldehyde, acetone, acrolein, hydrogen cyanide, hydroquinone, catechol, phenol, the cresols, the tobacco specific *N*-nitrosamines, nitrogen oxides, isoprene, 1,3-butadiene, furfural and other analytes were determined. For formaldehyde a slight but significant difference in total yield was detected (4.2 µg for the mentholated and 3.4 µg for the non-mentholated cigarette). No significant difference was observed in the yields of the vapor phase components, 1,3-butadiene, isoprene, acrylonitrile, benzene and toluene (342), or the yields of quinoline in the particulate phase (343). When the mainstream smoke vapor phase of menthol cigarettes was compared to non-menthol cigarettes, menthol cigarettes were found to have a significantly higher amount of 2-furfural (0.22 µg/cig) than non-menthol cigarettes (0.09 µg/cig). No difference was observed for furfural in mainstream particulate matter (0.15 µg/cig in both cigarettes) (344) or for the number of vapor phase free

radicals (345). Except for the menthol peak, the number of gas chromatographic peaks and their chromatographic response was comparable in the mainstream smoke of mentholated and non-mentholated cigarettes (346).

Contributing to a series of comprehensive studies coordinated by CARMINES (229), RUSTEMEIER *et al.* (230) analyzed the chemical composition of mainstream smoke from blended research cigarettes with and without additives. One of the additives tested was menthol applied at a rate of 1.8% to the tobacco in combination with casing materials consisting of corn syrup, licorice extract and cocoa shells. For technical reasons the menthol level applied (1.8%) was the same in the "low" and the "high" additive level cigarettes. The total particulate matter (TPM) of the test cigarettes was found to be higher than control (between 16% and 23%), presumably due to the increased transfer rates of additives and their pyrolysis products into smoke compared to the tobacco moiety of the filler.

Of the 51 smoke constituents analyzed, only formaldehyde, resorcinol and lead were markedly elevated, relative to TPM yield, while many others showed significant decreases - for tobacco specific *N*-nitrosamines around 30%. This was assumed to be the consequence of replacing tobacco by additives in the cigarette rod. If there was any adverse effect of added menthol on mainstream smoke composition, it did not stick out in this study. The findings were consistent with the lack of any increase of biological activity in the *in vitro* (231) and *in vivo* (232) assays done with the same test cigarettes.

A comprehensive overview of the effects of menthol on tobacco smoke properties was prepared in 2010 by HECK (267). HECK'S paper was intended to review available chemical, biological, toxicological and epidemiological studies; it is discussed in detail in the relevant sections of this review.

In 2011, GORDON *et al.* (347) investigated the effects of mentholization on cigarette mainstream smoke yields. Non-menthol cigarettes with a commercial full flavor blend ("tar": 17 mg, smoke nicotine: 1.3 mg) were mentholated at three different levels (0.10%, 0.20% and 1.40%); the original brand was used as control cigarette. For mentholization the authors used a new approach for the preparation of cigarettes, which differ only in menthol level. The basis of this technique is the volatility of menthol and its distribution between the surrounding atmosphere and a cigarette as described by BROZINSKI *et al.* in 1972 (284). Unmentholated cigarettes are exposed to menthol vapors for a defined period in a sealed vessel. We believe that this procedure is an excellent starting point for assessing the effects of menthol on cigarette smoke composition, and *in vitro* and in *vivo* toxicity.

The cigarettes were smoked according to the FTC regimen (142), which is practically identical to the corresponding ISO regulation (73). In comparison to the unmentholated control cigarette the amounts of nicotine, cotinine, *N*-nitrosonornicotine (NNN), 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK), pyrene, benzo[*a*]pyrene, and quinoline in mainstream smoke were largely unaffected by the menthol levels of the test cigarettes, as were the yields of selected mainstream smoke gas phase components (acetaldehyde, acetonitrile, acrylonitrile, benzene, isoprene, 1,3-butadiene and 2,5-dimethylfuran). These gas phase components were determined puff-by-puff using a proton transfer reaction mass spectrometer. This technique allows the real time determination of mainstream smoke components (348) before changes of smoke composition by ageing can take place. However, when testing a commercial cigarette, in which menthol was confined to a small portion of the filter by a capsule, the yield of the gas phase components appeared to be increased in the presence of menthol. In such cigarettes the menthol is set free by crushing the capsule in the filter. In our opinion, the crushing is a manipulation of the filter, which may change the physical properties of the filter and, therefore, its efficiency. This may be an explanation of the findings in the study.

The response of Altria Client Services (280) to the FDA concerning menthol use in cigarette manufacturing and the U.S. Industry Menthol Report (338) - both mentioned earlier - also provide extensive overviews of the effects of menthol on smoke composition and toxicity, smoking prevalence and the health of consumers. The U.S. Industry Menthol Report concluded (338, on page 233): "*The weight of the evidence clearly shows that the chemical compositions of the mainstream smoke from menthol and non-menthol cigarettes are very similar, apart from the presence of menthol itself*". In line with the Surgeon General´s framework for assessing causality (349), the Report determined (338, on page 79) that "*the evidence is suggestive of no causal relationship between the use of menthol in cigarettes and harmful changes in mainstream smoke chemistry*".

Based on the published literature and the reports of the cigarette industry to the FDA (280, 338) the TOBACCO PRODUCTS SCIENTIFIC ADVISORY COMMITTEE (TPSAC) concluded in 2011 (339, on page 218): "*The evidence is insufficient to conclude that it is more likely than not that menthol smokers inhale more smoke per cigarette or that they are exposed to higher levels of nicotine and other tobacco toxins.*" Addressing the issue of fine particles in the smoke of mentholated cigarettes the TPSAC stated (339, on page 210): "*The evidence is insufficient to conclude that smokers of menthol cigarettes face a different risk of tobacco-caused diseases than smokers of non-menthol cigarettes. Some toxicological studies raise concern, particularly the finding that the addition of menthol is associated with greater fine particles which are suspected to contribute to cardiovascular disease.*" The statement in the TPSAC report concerning the increase of the amount of fine particles in cigarette smoke by high levels of menthol is based on a "review" by LEE and GLANTZ (350) of publications by BAKER *et al.* (239), CARMINES (229) and RUSTEMEIER *et al.* (230). Unfortunately, the "review" is not really meaningful as "fine particles" are not discussed - and not even mentioned - in (at least) one of the three publications.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

Studies with the neat substance cannot provide sufficient evidence for its safety as tobacco additive. Synergistic effects with other tobacco or smoke components may create or enhance risks for smokers. Consequently, toxicological studies involving the matrix, tobacco, are called for.

In addition to the review of HECK (267), the available studies on the *in vitro* and *in vivo* toxicity of mainstream smoke from mentholated cigarettes were evaluated in the recently published contribution of ALTRIA CLIENT SERVICES (280) to the menthol discussion of the U.S. TOBACCO PRODUCTS SCIENTIFIC ADVISORY COMMITTEE, and the U.S. Industry Menthol Report (338). Both documents - in combination with relevant published studies, the conclusions reached in internal discussions and the presentations of committee members - were used for preparing the TPSAC report on menthol (339). In this document the effects of mentholation on the toxicity of cigarette mainstream smoke were also evaluated.

The mutagenic activity of mainstream smoke condensate from conventional cigarettes and a novel type of cigarettes, which heat rather than burn tobacco, both without and with menthol (1.03% in the tobacco blend according to (267)) was compared by AVALOS *et al.* (351) by means of the Ames assay. The *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 were used in this investigation with and without metabolic activation by the S-9 mix. The addition of menthol to tobacco was found to have no effect on mutagenic activity.

The same cigarette mainstream smoke condensates were used to evaluate the effect of menthol on cytotoxicity in Chinese Hamster ovary cells using the neutral red assay (352). No menthol related effect was observed.

The comparative sister chromatid exchange assay with smoke condensate of the cigarettes used in (351) and (352), conducted with Chinese Hamster ovary cells with and without metabolic activation with the S-9 mix, revealed no significant difference between menthol and non-menthol cigarettes (353).

ROEMER *et al.* (231) compared the mutagenic and cytotoxic effects of mainstream smoke of cigarettes containing menthol at a level of 1.8% in combination with casing materials consisting of corn syrup, licorice extract and cocoa shells. No differences compared to control were observed in the Ames test with *Salmonella* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 with and without S-9 metabolic activation. In the neutral red uptake assay, both the smoke particulate matter and gas phase of cigarettes containing the additives (including menthol) were somewhat less cytotoxic than those of cigarettes without additives.

Rare cases of mild skin sensitization by *l*-menthol were reported among smokers of mentholated cigarettes (354, 355).

In 1965, BOCK *et al.* (356) reported no difference in the specific tumorigenicity on mouse skin between the mainstream smoke condensates of non-mentholated and mentholated U.S. cigarettes. SCHIEVELBEIN (357) confirmed this report in a study with samples from the German market.

In 1999, GAWORSKI *et al.* (358) conducted a mouse skin painting tumor promotion bioassay with mainstream smoke condensates of cigarettes containing common flavoring additives, including menthol (0.5% in tobacco filler). Smoke condensate was applied after topical pre-treatment of the shaved dorsal skin of SENCAR mice with 50 µg 7,12-dimethylbenz[*a*]anthracene (DMBA) dissolved in 0.1 mL acetone. The mentholated test cigarettes showed no significant difference compared to the control cigarettes in any parameters of tumorigenic response, such as percentage of tumor bearing animals, tumor latency and tumor multiplicity.

The influence of menthol on the biological activity of mainstream smoke was investigated as part of the development of a novel cigarette type, which heats rather than burns tobacco. COGGINS *et al.* (359) conducted a subchronic 90-day nose-only inhalation study with Sprague-Dawley rats comparing the histopathological response to mainstream smoke from the traditional and the novel cigarette type. The animals were exposed to three different doses of wet particulate matter, one hour per day, 5 days per week for 13 weeks. The highest dose was 0.64 mg per liter of air. As expected, histopathological changes (mucus-secreting cells; nasal, laryngeal, and bronchial hyperplasia and squamous metaplasia, pulmonary macrophages) were absent or (primarily in the larynx) substantially reduced and completely reversible in the animals exposed to the smoke of the novel cigarette type. In an inhalation study of similar experimental design (360) with mentholated cigarettes of the traditional and the novel type, the response pattern observed after smoke exposure and the reversibility of lesions were the same as with the comparable non-mentholated test pieces (359). The addition of menthol did not influence the substantial difference in biological effects noted between the two types of cigarettes.

A 13-week comparative nose-only smoke inhalation toxicity study was conducted by GAWORSKI *et al.* (361) in male and female Fischer 344 rats using a U.S. style filter cigarette without menthol and a similarly blended cigarette made with tobacco containing 0.5% synthetic *l*-menthol. The animals were exposed 13 weeks for 1 hour/day and 5 days/week to target mainstream smoke particulate concentrations of 200, 600 or 1,200 mg/m³, while reference rats were exposed to filtered air. The internal dose biomarkers, carboxyhemoglobin, serum nicotine and serum cotinine, indicated comparable exposure to the test and control cigarettes. The effects typically noted in rats exposed to high levels of cigarette mainstream smoke were similar for both cigarette types (reduced body weight, increased heart-to-body weight ratio and lung weight and histopathological changes in the respiratory tract). Rats exposed to the smoke of the control cigarette displayed a dose-related increase in nasal discharge that was not observed in rats exposed to the smoke of the mentholated cigarettes. All smoke-related effects diminished significantly during a 6-week non-exposure recovery period. The authors concluded that the addition of 0.5% menthol to tobacco had no substantial effects on the character or extent of the biological responses in rats normally associated with the inhalation of cigarette mainstream smoke.

In 1998, GAWORSKI *et al.* (362) reported a quite similarly designed subchronic 13-week nose-only inhalation study in male and female Fischer 344 rats and found that the mainstream smoke of test cigarettes containing various (undefined) "representative combinations" of 172 flavoring additives "*had no discernible effect on the character or extent of the biologic responses normally associated with inhalation of mainstream cigarette smoke in rats*". Synthetic *l*-menthol at a level of 0.5% on the processed tobacco was included in the study.

As part of the comprehensive study on the effects of additives on cigarette mainstream smoke, coordinated by CARMINES (229), VANSCHEEUWIJCK *et al.* (232) compared in a 90-day subchronic rat inhalation study the response elicited by the mainstream smoke of cigarettes containing 1.8% *l*-menthol (combined with rather high levels of corn syrup, licorice extract and cocoa shells) to a matched control cigarette without any additives. Groups of 10 male and 10 female Sprague-Dawley rats were exposed every day for 6 hours to 150 µg total smoke particulate matter/liter air, followed by a 42-day post-inhalation period. It should be noted that, in this study, smoke exposure concentrations were considerably lower and smoke exposure times much longer than in many earlier inhalation studies in order to increase test sensitivity and avoid the artifacts of an excessively high TPM exposure regimen. The control group consisted of 14 male and 14 female animals. No significant differences in respiratory rate and volume, body weight gain, clinical chemical and hematological parameters (such as blood nicotine and carboxyhemoglobin, and the relative distribution of nicotine metabolites) and gross pathology, were observed between the additive (including menthol) containing and the additive free cigarettes. The comprehensive examination of smoke related histopathological effects in the respiratory tract found no notable differences in character or severity attributable to the additives (including menthol) in the test cigarettes. VANSCHEEUWIJCK *et al.* concluded that the toxicity of the smoke of menthol containing test cigarettes, as used in the study, did not differ in any substantial way from the control cigarettes.

ALTRIA CLIENT SERVICES (280) and the U.S. Industry Menthol Report (338) also concluded in their evaluations of the scientific literature that menthol in cigarettes did not adversely affect the toxicological properties of cigarette mainstream smoke.

In summary, the results of all toxicological studies presented above are consistent with the conclusion that mentholated cigarettes - with quite different levels of menthol added to tobacco - are not likely to increase the hazard of smoking compared to non-menthol cigarettes.

4.5.2. Glycerol

- Use and toxicological assessment

Glycerol has been identified as a natural constituent of oriental tobacco (0.34–0.39%), flue cured tobacco (0.27–0.32%) and Burley tobacco (0.07–0.12%) (363). Due to its hygroscopic properties it is used as a humectant in tobacco products, commonly combined with 1,2-propylene glycol.

Glycerol is considered to have low acute oral toxicity. An oral LD_{50} of around 25 g/kg was found in rats (364, 365). The oral LD_{50} in rabbits was 27 g/kg (366). Non-lethal effects were observed in small rodents by several authors after the application of high doses of glycerol (367–372).

The toxicity of glycerol was evaluated thoroughly by the R.J. Reynolds Tobacco Co. in the course of the development of a cigarette that heats instead of burns tobacco (373). Relevant extensively documented literature search batteries were used focusing on several *in vitro* assays and animal tests.

The genotoxic potential of glycerol was evaluated in an *in vitro* test battery by DOOLITTLE *et al.* (374). It included the Ames *Salmonella typhimurium* assay with the strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538, the rat hepatocyte unscheduled DNA synthesis assay, the Chinese hamster ovary (CHO) chromosome aberration assay, the CHO sister chromatid exchange assay and the CHO mammalian mutagenesis assay. These tests - except the unscheduled DNA synthesis assay - were conducted with and without metabolic activation by the rat liver S-9 mix. The results of all tests were negative. Neither glycerol nor its (experimental) metabolites showed genotoxic activity in the test battery used. This is confirmation of the earlier assessment of the U.S. FOOD AND DRUG ADMINISTRATION (375) that glycerol was not mutagenic.

The toxic effects of inhaled glycerol in Sprague-Dawley rats by nose-only exposure for 2 weeks (1.0, 1.9 and 3.9 mg glycerol/liter air) and 13 weeks (0.03, 0.16 and 0.66 mg glycerol/liter air) were investigated by the R.J. Reynolds Tobacco Co. together with Battelle Northwest Laboratories (376, 377). The major finding was reduced body weight gain when more than 1 mg glycerol per liter of air was inhaled by the animals for two weeks. Minimal histopathologic changes but no biologically significant effects were observed. Following daily exposure to aerosol concentrations of 1.0, 1.9 and 3.9 mg/liter mild squamous metaplasia of the epithelial lining at the base of the epiglottis was observed in the 2-week study at all concentrations. In the 13-week study, when rats were exposed to concentrations between 0.03 and 0.66 mg glycerol/liter air, mild squamous metaplasia was seen only at the highest exposure level.

GUERRANT *et al.* (378) reared six generations of rats on diets containing a dose of 5 g/kg/day glycerol without significant adverse effects on the offspring. WEGENER (379) administered 2 g/kg/day of glycerol to male and female rats during a two generation reproduction study and observed no adverse effects on reproduction or the growth and development of the offspring.

Investigators of the U.S. FOOD AND DRUG RESEARCH LABORATORIES (380) administered up to 1.28 g/kg/day of glycerol orally to pregnant mice on gestation days 6 through 15, and up to 1.31 g/kg/day to pregnant rats with no effects on nidation or maternal or fetal survival. VERRETT *et al.* (381) demonstrated no teratogenic effects of glycerol in the chicken embryo test.

In summary, based on the results of the studies presented above it is expected that neat glycerol in doses as inhaled by smoking cigarettes (approximately 1 to 2 mg per cigarette) is not harmful for humans.

- Inclusion level in cigarettes, transfer and pyrolysis

Glycerol is typically applied to tobacco at levels up to 2.5% (21), generally in combination with 1,2-propylene glycol. The German Tobacco Ordinance (TVO) stipulates that the maximum inclusion level of total humectants (in practice, glycerol and 1,2-propylene glycol) in cigarettes is 5% by weight (266).

Adding ^{14}C-labeled glycerol to cigarette tobacco, LARSON and HARLOW (382) reported that 81.9% of the activity transferred to mainstream smoke was found in the particulate phase, and the rest in the gaseous phase as labeled

carbon dioxide (8.8%), carbon monoxide (6.0%) and other trace components, such as carbonyls. The occurrence in cigarette sidestream smoke of labeled compounds generated from glycerol during smoking was also reported by LARSON and HARLOW.

According to a study of LAURENE *et al.* in 1965 (383), 5.27% of the glycerol added to tobacco in plain cigarettes was transferred into mainstream smoke. KOBASHI *et al.* (384) found that the transfer rate of glycerol from tobacco into the mainstream smoke of filter and non-filter cigarettes was 12% and 14%, resp. BEST (385) observed 10% transfer into mainstream smoke using a conventional filter cigarette and ^{14}C-labeled glycerol.

Summarizing several R.J. Reynolds research documents RODGMAN (21) concluded that about 7% of the glycerol, present on tobacco of filter cigarettes with a "tar" level of around 16 mg under ISO standard conditions (73), was transferred into mainstream smoke. The transfer rate depended on the type and construction of the cigarettes.

LIU (386) investigated the effect of different levels of glycerol added to tobacco (up to 11.4%) on cigarette mainstream smoke yields. The transfer of glycerol was generally found to be proportional to the glycerol level in tobacco. The proportion of glycerol in the "tar" of a ventilated king size filter cigarette ("tar" level 12.5 mg) was as high as 36% for a blend with 11.4% added glycerol.

Based on the results obtained in pyrolysis experiments BAKER and BISHOP (199) estimated that glycerol added to tobacco was transferred into mainstream smoke more than 99% intact.

Glycerol may produce degradation products when subjected to elevated temperatures. The generation of acrolein and acetaldehyde from neat glycerol was shown in 1983 in pyrolysis studies in the presence of steam at 650–750 °C (387). The possible contribution of glycerol to the formation of these aldehydes in tobacco smoke was one of the reasons for the intense scrutiny of glycerol as a tobacco additive.

Using different experimental conditions, CARMINES and GAWORSKI (388) subjected in 2005 neat glycerol to pyrolysis in air at simulated tobacco burning temperatures up to 900 °C. Glycerol did not pyrolyze extensively suggesting that glycerol may be transferred largely intact to mainstream smoke; acrolein and glycolaldehyde appeared to be minor pyrolysis products under these experimental conditions. Less than 1% of the glycerol pyrolyzed appeared to be converted into the two aldehydes.

GAGER *et al.* (389) studied the generation of acrolein in mainstream smoke with cigarettes containing ^{14}C-glycerol. Under FTC standard smoking conditions (142), which are practically identical with the ISO smoking regimen (73), about 0.1% of the radioactivity in smoke was recovered as acrolein. This result suggested that added glycerol was a minor precursor for acrolein in mainstream smoke.

In 1977, a study of the U.S. NATIONAL CANCER INSTITUTE (68) showed no difference in acrolein levels in the mainstream smoke of experimental cigarettes made with and without 2.8% glycerol.

- Attractiveness and addictiveness

Glycerol has a sweet taste. However, the levels used in cigarette manufacturing do not result in a "sweet" taste of the smoke, which might make glycerol containing cigarettes more attractive to some smokers. In addition, as pointed out by SCENIHR in 2010 (92), glycerol per se - like all other additives used for cigarettes - has no addictive potential in humans nor does it enhance the addictiveness of tobacco smoke components. Glycerol is an additive used for maintaining tobacco in good condition during manufacturing and after cigarette purchase by consumers.

- Effect on cigarette mainstream smoke composition

One of the first studies of the effects of glycerol added to tobacco on cigarette mainstream smoke composition was published by DE SOUZA and SCHERBAK in 1964 (390). It was reported that the addition of up to 6% glycerol to cigarette tobacco had no effect on mainstream smoke benzo[*a*]pyrene levels.

The effect of glycerol in combination with other tobacco additives (1,2-propylene glycol and a large number of different casing and top flavoring materials) on the composition of cigarette mainstream smoke was studied by RUSTEMEIER *et al.* (230). Target concentrations for glycerol were 2.8% and 4.2% in the test cigarettes. A battery of 51 smoke components was determined. Total particulate matter (TPM) of the test cigarettes was higher (by 17% and 28%, resp.) compared to the additive free control cigarettes. Relative to TPM a decrease in nicotine, nitric oxides, formaldehyde, phenols, acrylonitrile, naphthalene, tobacco specific *N*-nitrosamines and arsenic was observed in the mainstream smoke of additive containing cigarettes. However, the observed effects cannot be attributed directly to specific tobacco additives, including glycerol. It is primarily an effect of the dilution of the tobacco in the cigarettes and the smoke generated in the presence of the additives.

Applying glycerol as the only tobacco additive, CARMINES and GAWORSKI (388) investigated its effect on mainstream smoke constituents. Besides "tar", nicotine, water and carbon monoxide, 33 individual components were determined in the mainstream smoke of filter cigarettes (30% tip ventilation) with target levels of 5%, 10% and 15% glycerol in the blend (actual levels were 3.2%, 6.2% and 8.4%). For control, a cigarette with an identical tobacco blend and no added glycerol was used. Nicotine in mainstream "tar" was significantly decreased in cigarettes with 10% and 15% glycerol. Relative to "tar", 10% and 15% glycerol resulted in a statistically significant increase of acrolein (by 9%) and a decrease of formaldehyde, acetaldehyde, propionaldehyde, aromatic amines, nitric oxide and *N*-nitrosamines. No effect of added glycerol was observed on hydrogen cyanide, the polycyclic aromatic hydrocarbons and the gaseous components, acrylonitrile, 1,3-butadiene and isoprene. The addition of 5% glycerol to the tobacco produced the decrease of the same smoke constituents as the addition of 10% or 15%, but there was no concomitant increase in acrolein. This is an important finding in view of the fact that 5% represents the application level of humectants - including glycerol - generally not exceeded in cigarettes.

In 2010, YIP *et al.* (391) investigated the formation of acrolein in mainstream smoke from ^{13}C-labeled glycerol in cigarettes of different design (additive free filter cigarettes with ISO "tar" yields of 5, 10 and 14 mg/cig and 2.5–3.0%

(w/w) labeled glycerol in the blend). Two different machine smoking regimes - FTC (142) and Canadian Intense (74) - were used. They found that less than 0.1% of the added glycerol was converted to acrolein in mainstream smoke for all cigarette designs and smoking regimes studied.

The absence of substantial effects of glycerol added to a cigarette blend on mainstream smoke composition was confirmed in the tobacco additives study initiated by the German regulatory authorities (176–178).

Humectants, such as glycerol, may influence the hygroscopic properties and growth of smoke particles in cigarette mainstream smoke (392, 393) and, consequently, the deposition of these particles in the human respiratory tract (394). MOLDOVEANU and COLEMAN (395) investigated the influence of glycerol on the retention of mainstream smoke particles by humans using solanesol as a marker. On average, 69.5% (SD = 9.4%) of mainstream smoke solanesol was retained by smokers of a commercial cigarette without any additives and a natural glycerol content of 0.19%, compared to an average retention of 69.4% (SD = 10.5%) from an identical cigarette with 2.3% glycerol added to the tobacco. The authors concluded that the addition of 2.3% glycerol, which is within the inclusion range of this humectant in commercial cigarettes, had no influence on solanesol retention and, in consequence, the retention of smoke particles.

The results of the studies presented above suggest that adding glycerol to cigarette tobacco at use levels typical for commercial cigarettes does not adversely alter mainstream smoke chemistry or influence particle retention in humans.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

In 1977, the U.S. NATIONAL CANCER INSTITUTE (68) evaluated the mainstream smoke of experimental cigarettes made without or with 2.8% glycerol added to the tobacco. The smoke of the glycerol containing test cigarettes was found to be modestly lower in ciliastatic potency than the glycerol free control cigarettes, determined by the extent of ciliar transport inhibition caused by repeated exposure of chicken tracheal epithelium to mainstream smoke. Similarly, a cytotoxicity bioassay measuring the ability of mainstream smoke to inhibit the growth of mammalian cells *in vitro* (a KB tumor cell culture) showed a modestly lower effect of glycerol containing cigarettes compared to control.

WILSON *et al.* (396) investigated the local and systemic carcinogenicity in mice of cigarette mainstream smoke condensate spiked with two doses of glycerol (17.5% and 35%). The addition of glycerol reduced the incidence of benign and malignant tumors as well as the development of hyperplasia normally seen after repeated application of cigarette smoke condensate to mouse skin. There was no change in the incidence of systemic effects (e.g., tumors of other organs besides skin) attributable to cigarette smoke condensate with added glycerol.

In our opinion, the observed reduced incidence of tumorigenicity is caused by the dilution of the condensate with glycerol. This demonstrates as well that there is no synergistic effect concerning tumorigenicity between cigarette smoke condensate and glycerol in mice.

ROEMER *et al.* (231) evaluated the cytotoxicity and genotoxicity of the mainstream smoke of cigarettes after glycerol was added to tobacco in different concentrations in a mixture with other additives (flavors and casing materials). No increase in the cytotoxicity of the gaseous and particulate phases (neutral red uptake by mouse embryo BALB/c3T3 cells) or the genotoxicity of the particulate phase (Ames assay) was observed with the experimental cigarettes compared to control.

The *in vitro* toxicological properties of additive containing test cigarettes manufactured at the request of the German regulatory authorities were evaluated by ROEMER *et al.* (177). The cytotoxicity (neutral red uptake) of the total particulate matter of glycerol containing cigarettes was decreased by approximately 15% compared to cigarettes with no added glycerol. The mutagenicity of mainstream smoke total particulate matter in the *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 was not affected by the addition of glycerol to the tobacco filler.

The effects of the mainstream smoke condensate of glycerol containing cigarettes was evaluated in a skin painting bioassay with female SENCAR mice by GAWORSKI *et al.* (358). In female SENCAR mice, initiation was done topically with 50 µg 7,12-dimethylbenz[*a*]anthracene (DMBA) dissolved in 0.1 mL acetone, followed by promotion three times a week for 26 weeks with either 10 or 20 mg of cigarette smoke condensate of test cigarettes containing 2.4% glycerol or control cigarettes with no glycerol added. While incidence, latency and multiplicity data of tumors in some cases differed between test and control cigarette smoke condensate, all effects appeared to be within the normal variation for the model system, SENCAR mouse. The authors concluded that the addition of glycerol to cigarettes did not increase tumorigenicity in the skin painting assay.

In 2002, HECK *et al.* (397) investigated the effects of glycerol and 1,2-propylene glycol in cigarettes on mainstream smoke in a subchronic inhalation study. Fischer 344 rats were exposed nose-only for 13 weeks. American blend filter cigarettes were used containing either glycerol added at 5.1% to the tobacco blend, 1,2-propylene glycol at 2.2% or combinations of these humectants totaling 2.3%, 3.9% and 7.2%. Other groups of rats were exposed similarly to the smoke of control cigarettes without added humectants or to filtered air (sham control). The well known effects of cigarette smoke exposure were observed in the animals (e.g., reductions in body weight, occasional increases in heart and lung weights, etc.). No significant differences were seen in the biochemical data (carboxyhemoglobin, blood serum nicotine and cotinine, increase in serum alkaline phosphatase, decrease in serum glucose, etc.) between the humectant containing and the control cigarettes. There was also no difference in respiratory tract histopathology. The smoke related changes eased off substantially during the 6-week post-exposure recovery period. The authors concluded that the addition of glycerol and 1,2-propylene glycol to tobacco, separately and in combination, had no remarkable effects on the site, occurrence or severity of respiratory tract changes or the

measured indices of pulmonary function. The addition of these humectants did not significantly affect the biological activity of inhaled cigarette smoke in this rat model.
VANSCHEEUWIJCK *et al.* (232) reported comparable effects of mainstream smoke from cigarettes with and without additive mixtures containing different amounts of glycerol on the hematology, gross pathology or histopathology of the upper respiratory tract of rats in a 90-day nose-only inhalation study.
BAKER *et al.* (239) compared the mainstream smoke of cigarettes containing a mixture of additives - including 7% glycerol on tobacco - with additive free cigarettes in a 90-day inhalation study with rats. No difference in overall toxicity (analyzing histopathological lesions in the upper respiratory tract) was seen.
CARMINES and GAWORSKI (388) evaluated the influence of glycerol on the *in vitro* and *in vivo* toxicity of cigarette mainstream smoke. The actual levels of glycerol in the test cigarettes were 3.2%, 6.2% and 8.4%. The lowest level corresponded approximately to what is used in commercial cigarettes. Biological *in vitro* tests (Ames assay with different strains of *Salmonella typhimurium*, neutral red uptake, micronucleus test) indicated no relevant differences in the cytotoxic or genototoxic potential of mainstream smoke of cigarettes with added glycerol compared to control cigarettes. Nose-only exposure of rats in a 90-day inhalation study with mainstream smoke of cigarettes containing 8.4% glycerol did not produce any adverse effects in the animals compared to control. The authors concluded that adding glycerol to cigarette tobacco at typical use levels did not modify the biological effects normally associated with the exposure to mainstream cigarette smoke.

4.5.3. 1,2-Propylene glycol

- Use and toxicological assessment

The use of 1,2-propylene glycol in tobacco products, cosmetics and various other consumer products is supported by numerous toxicological studies involving several *in vitro* assays and animal studies.
FLORIN *et al.* (398) evaluated the mutagenicity of neat 1,2-propylene glycol in the Ames assay with the *Salmonella typhimurium* strains TA 98, TA 100, TA 1535 and TA 1537, with and without metabolic activation with the S-9 liver microsome fraction of rats. No mutagenic activity was observed. 1,2-Propylene glycol produced no response in the sister chromatid exchange (SCE) test (399).
An overview of the toxicity of propylene glycol was prepared by LAKIND *et al.* (400). The overview was focused on oral, inhalatory and dermal routes of exposure. Propylene glycol had low acute toxicity and localized dermal effects were mild. The data suggested that propylene glycol may have skin contact sensitization potential. Exposure in laboratory animals was associated with reversible hematological changes. Unfortunately, the authors provided no information concerning the isomer(s) of propylene glycol reviewed for toxicity. Two different structures of this compound exist (1,2-propylene glycol and 1,3-propylene glycol), which may have different toxicological properties.
SUBER *et al.* (401) exposed groups of male and female Sprague-Dawley rats nose-only to 0.16, 1.0 and 2.2 mg of propylene glycol per liter of air for 6 hours per day, 5 days per week for 13 weeks. According to the authors, neat propylene glycol (presumably the 1,2-isomer) administered by inhalation did not show signs of systemic toxicity in rats at the doses used. Exposure to 2.2 mg/liter affected the nasal passages by acting as an astringent for the respiratory epithelium; however, these changes were transient and not considered adverse effects.
The potential human reproductive and developmental effects of 1,2-propylene glycol were evaluated by an expert panel of the U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICE (402). Data of a multi-generation study had shown that 1,2-propylene glycol had no reproductive toxicity in male and female mice with exposures up to 5% in drinking water over an 18-week period (one week prior to cohabitation, 14 weeks during cohabitation and 3 weeks after cohabitation) or in progeny. While no information was available from human studies the mouse data were judged to be relevant for the consideration of risk in humans. In a "weight-of-evidence" approach the panel concluded that 1,2-propylene glycol was not expected to have adverse effects on human reproduction and development.

- Inclusion level in cigarettes, transfer and pyrolysis

American blend cigarettes may contain up to 2% 1,2-propylene glycol (21), generally in combination with glycerol. The German Tobacco Ordinance (TVO) stipulates that the maximum inclusion level of total humectants (in practice, glycerol and 1,2-propylene glycol) in cigarettes is 5% by weight (266).
A transfer rate of 4.5% for 1,2-propylene glycol into cigarette mainstream smoke was reported by LAURENE *et al.* (383). KOBASHI *et al.* (384) found that 12.6% of 1,2-propylene glycol in plain cigarettes was transferred unchanged to mainstream smoke; in filter cigarettes the rate was 9.9%.
On the basis of several R.J. Reynolds research documents RODGMAN (21) concluded that between 4 and 10% of 1,2-propylene glycol was transferred into mainstream smoke.
In line with their pyrolysis studies BAKER and BISHOP (199) assumed that 1,2-propylene glycol applied to cigarette tobacco was transferred into mainstream smoke more than 85% intact. Small amounts of the isomer 1,3-propylene glycol, acetol (hydroxyacetone), acetic anhydride and pyruvaldehyde were found in pyrolysate.

- Attractiveness and addictiveness

Like glycerol, 1,2-propylene glycol has neither addictive potential *per se* nor does it increase the addictiveness of tobacco products (92). Its use as a humectant is - like glycerol - a measure of quality assurance in cigarettes but not of enhancing the attractiveness of a specific brand.

- Effect on cigarette mainstream smoke composition

The effect of 1,2-propylene glycol on cigarette mainstream smoke composition in combination with various other flavor and casing additives was evaluated by RUSTEMEIER

et al. (230). Besides an increase of mainstream total particulate matter the decrease of several smoke constituents was observed in the smoke of the 1,2-propylene glycol containing cigarettes. This is discussed above in the section on glycerol.

The effect of 1,2-propylene glycol on cigarette mainstream smoke composition was evaluated by BAKER *et al.* (208). The addition of 8.33% 1,2-propylene glycol to the tobacco blend of a test cigarette showed only one small statistically significant increase (by 12%) of the mainstream smoke yield of a "Hoffmann analyte", namely propionaldehyde. It should be pointed out that 8% 1,2-propylene glycol in a cigarette blend is several times the level of this humectant in modern commercial cigarettes.

In 1999, KAGAN *et al.* (403) reported a propylene oxide level of 370 ng/g tobacco in the mainstream smoke of two commercial non-filter cigarettes and postulated that 1,2-propylene glycol was the precursor for this epoxide. Propylene oxide was also measured by GORDON and COLEMAN (404), who found 0.89 and 1.1 µg/cig in the mainstream smoke of the Kentucky reference cigarettes 2RF4 using two different experimental gas chromatographic setups and two different smoking machines. This result was confirmed by DIEKMANN *et al.* (405), who measured 0.93 and 0.65 µg/cig in the mainstream smoke of the Kentucky reference cigarettes 1R4F and 2R4F, resp., using a new rapid GC-MS method for smoke analysis. In a comprehensive toxicological study with 1,2-propylene glycol, 591 ng/cig propylene oxide were measured in the smoke of additive free cigarettes and 7,329 ng/cig when the manufactured product contained the unusually high level of almost 8% of 1,2-propylene glycol in the tobacco (406).

Because of its extensive use in the chemical and food manufacturing industries propylene oxide and its biological effects were investigated in several studies. BOOTMAN *et al.* (407) evaluated the mutagenic activity of propylene oxide in both bacterial and mammalian cell systems and *in vivo* in mice. Propylene oxide induced base-substitution mutations in spot test with strains of *Salmonella typhimurium* and *Escherichia coli* and produced reversions dose-dependently in the Ames assay with *Salmonella typhimurium* TA 100 and TA 1535 and in liquid culture with strain TA 100. Chromosomal aberrations were seen in cultured dividing human lymphocytes. The *in vitro* activity of propylene oxide was, however, not reproducible in intact animals. Even high does administered orally to mice produced no response in a micronucleus assay or a dominant lethal test. Rapid metabolic conversion of propylene oxide to propylene glycol may be a reason for these observations.

The chronic inhalation toxicity and carcinogenicity of propylene oxide were studied in male and female Wistar rats by KUPER *et al.* (408). Atmospheres with 0, 30, 100 or 300 ppm propylene oxide were inhaled for 6 hours/day, 5 days/week and 28 months (with 100 animals of each sex per group). Increased incidences of degenerative and hyperplastic changes of the nasal mucosa were observed in all exposed groups. Tumor incidence was elevated only in the 300 ppm group (both benign and malignant mammary tumors in female animals; the total number of rats bearing malignant tumors at sites other than the mammary glands in both sexes).

The available studies of the mutagenicity and carcinogenicity of propylene oxide and the current efforts to develop molecular dosimetry methods for propylene oxide DNA adducts were comprehensively reviewed by RÍOS-BLANCO *et al.* (409). The paper also reported the analysis of *N*7-guanine adducts of propylene oxide (*N*7-(2-hydroxypropyl)guanine) in respiratory, olfactory and hepatic tissues of male F344 rats following 4-week inhalation exposure to 500 ppm.

In 2002, KOLMAN *et al.* (410) reviewed new data (published in 1990–2001) on the genotoxic effects of ethylene oxide, propylene oxide and epichlorohydrin in humans. The use of DNA and hemoglobin adducts as biomarkers of exposure to propylene oxide was discussed as were recent *in vitro* data on genotoxic effects induced in mammalian cells. According to IARC propylene oxide is classified as "possibly carcinogenic to humans" (Class 2B) (411). However, cancer epidemiology data are lacking for propylene oxide.

The question whether propylene oxide in cigarette smoke gives reason for toxicological concern was addressed in two investigations with the focus on inhalation studies. HECK *et al.* (397) examined cigarettes spiked with glycerol and 1,2-propylene glycol (singly and in combination) and found that the addition of these humectants to cigarettes did not significantly affect the biological activity of inhaled cigarette smoke in rats.

GAWORSKI *et al.* (406), in the study mentioned before, subjected the smoke of test cigarettes with target levels of 4, 7 and 10% 1,2-propylene glycol added to tobacco to chemical analysis (41 analytes), toxicological *in vitro* assays (bacterial mutagenicity and cytotoxicity using neutral red uptake) and a sub-chronic inhalation study. The addition of 1,2-propylene glycol reduced the concentrations of some smoke components (e.g., nicotine), but had minimal effects on the biological responses compared to the control cigarette without the humectants. An effect of smoke on the biological endpoints that could have been attributed to propylene oxide was not seen.

In an experiment comparable to the one performed with glycerol (395), MOLDOVEANU and COLEMAN (412) investigated the particle retention in humans from cigarette mainstream smoke under the influence of 1,2-propylene glycol. Retention from a control cigarette with no 1,2-propylene glycol on average was 72.5% (SD = 11.7%) and from a cigarette with 3% 1,2-propylene glycol added on average 70.8% (SD = 14.5%). The study indicated no significant difference in particle retention between the two cigarettes.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

ROEMER *et al.* (231) evaluated the effect of mixtures of tobacco additives on cigarette mainstream smoke cytotoxicity and mutagenicity. In combination with other flavor and casing materials the cigarettes contained between 0.5% and 3.3% 1,2-propylene glycol on the tobacco. No significant differences in cytotoxicity and mutagenicity were observed for mainstream smoke between the experimental and control cigarettes.

Mainstream smoke of the same cigarettes as used in the study of ROEMER *et al.* (231) was tested in a subchronic 90-day nose-only inhalation study in rats by VANSCHEEUWIJCK *et al.* (232). An increase of mainstream smoke toxicity due to the use of 1,2-propylene glycol was not seen.
Examining the effects of the humectants, glycerol and 1,2-propylene glycol added to tobacco, on mainstream cigarette smoke in a subchronic 13-week nose-only inhalation study with rats (described above in more detail), HECK *et al.* (397) found no significant difference between the animal groups exposed to smoke from cigarettes with and without added humectants.
Comparable results were obtained by BAKER *et al.* (239) in their evaluation of the effects of tobacco additives on cigarette mainstream smoke toxicity using test cigarettes containing up to 8.3% 1,2-propylene glycol applied on tobacco in a casing mixture. They also studied the effects of this humectant on toxicity in several *in vitro* and *in vivo* assays.
The studies presented above (231, 232, 239, 397) had applied 1,2-propylene glycol to cigarette tobacco in a casing formula or in combination with glycerol. Therefore, overlapping effects with other additives could have influenced the results. In contrast, GAWORSKI *et al.* (406) used for their study a series of experimental cigarettes containing different target levels solely of 1,2-propylene glycol between 4% and 10% and compared them with similar cigarettes with no 1,2-propylene glycol added. The main toxicological comparison was based on a sub-chronic nose-only inhalation study with mainstream smoke in Sprague-Dawley rats (exposure to 150 µg total particulate matter/liter air, 6 hours per day for 90 consecutive days). In addition, the mutagenicity of the particulate phase was evaluated by the Ames assay with five *Salmonella* strains, with and without metabolic activation, and the cytotoxicity of both particular and vapor phase by the neutral red uptake assay. Minimal or no effects on the biological responses in both the *in vitro* tests and the inhalation study were observed following the addition of 1,2-propylene glycol. Most of the changes produced in the 90-day nose-only exposure in rats were resolved in the 42-day post-inhalation period. From the results of the studies presented and discussed above it is concluded that 1,2-propylene glycol as used in tobacco products does not increase the overall toxicity of cigarette mainstream smoke.

4.5.4. Sorbitol

- Use and toxicological assessment

D-Sorbitol is used as sweetener or humectant in foodstuffs, pharmaceutical preparations, toothpaste and cosmetics. It is also used in tobacco products, such as cigarettes.
In 1967, STAPLES *et al.* (413) studied the effects of sorbitol on the gastrointestinal mucosa of rats and dogs. After repeated oral administration of doses of more than 3.0 g/kg body weight weak irritating effects were observed in both species. These effects were less severe than produced by glycerol at the same dose.
MACKENZIE *et al.* (414) administered sorbitol in high doses in the diet to three successive generations of male and female Charles River BR rats. Sorbitol levels up to 10% had no adverse effects on growth or reproductive performance in either sex.
The relevant toxicological publications and data concerning D-sorbitol can be found in the WHO FOOD ADDITIVE SERIES No. 13: Sorbitol (415). Sorbitol was administered in the diet of beagle dogs, rats and rabbits. No severe toxic effects were documented.

- Inclusion level in cigarettes, transfer and pyrolysis

Sorbitol appears on the list of "Permitted additives to tobacco products in the United Kingdom" (416) with a maximum level of 8.0% (w/w) in cigarette tobacco.
No data were published on the transfer of sorbitol into smoke but it is expected that minute amounts of this hexavalent alcohol may be found in cigarette smoke similar to glucose and fructose (417). However, this assumption was not confirmed by the pyrolysis study of BAKER and BISHOP (199). In this study, mimicking the conditions in the burning cone of a cigarette during puffing, neat sorbitol broke down completely generating furfural as the main degradation product (31.4%) and various other furan derivatives. The presence of furfural in tobacco and tobacco smoke has been known for more than a century (418). It was assumed that pentosanes in tobacco were the precursors.

- Attractiveness and addictiveness

The taste of the polyalcohol sorbitol is intensively sweet, comparable to sugar. In cigarette manufacturing sorbitol is used as a humectant, commonly in combination with glycerol and/or 1,2-propylene glycol. Due to its polarity there is - if any - only a minute transfer of sorbitol from tobacco into mainstream smoke. Therefore, the sweetness of sorbitol cannot be noticed in smoke. The assumption that sorbitol increases cigarette attractiveness for specific groups of smokers is more than unlikely. According to SCENIHR (92) there is also no effect of sorbitol on the addictive potential of tobacco products.

- Effect on cigarette mainstream smoke composition

The amount of furfural in cigarette mainstream smoke, generated from sorbitol when used as tobacco humectant, is not precisely known but seems to be extremely low. When test cigarettes containing 3.5% sorbitol in the tobacco blend were smoked 6.4 µg/cig furfural was found in the particulate phase, and 3.6 µg/cig in the gas phase of mainstream smoke, a level not significantly different from sorbitol free control cigarettes (199, 239). Obviously, sorbitol did not decompose during cigarette smoking the same way it did in pyrolysis studies. This is another example of the false conclusions that may be drawn using data of inexpedient pyrolysis studies; it indicates that pyrolysis techniques are not generally suitable for predicting the fate of non-volatile substances in a burning cigarette.
The effect of sorbitol on the composition of cigarette mainstream smoke was recently studied by COGGINS *et al.* (242). The smoke of cigarettes with three different levels of sorbitol (1.5%, 4.5% and 10%) was investigated. In com-

parison to the additive free control cigarette reductions were noted for several smoke components such as nicotine, the tobacco specific nitrosamines and other nitrogen containing substances. According to the authors, these reductions may be due to the replacement of tobacco by sorbitol.
A review of the effects of humectants, including sorbitol, on cigarette mainstream smoke was prepared by RODGMAN (21); see Section 4.3. on page 429.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

In 1979, SATO *et al.* (419) reported a reduction of the specific mutagenicity of mainstream smoke of cigarettes containing sorbitol in the Ames assay with and without metabolic activation. The effect was more pronounced in strain TA 98 than TA 100 after metabolic activation. Without activation no reduction was observed. The exact amount of sorbitol in the test cigarettes cannot be identified using the data reported by SATO *et al.*
The effects of sorbitol - as part of an experimental mixture of tobacco additives - on cigarette mainstream smoke were investigated by BAKER *et al.* (239). No meaningful difference in *in vitro* and *in vivo* toxicity was detected between sorbitol containing and sorbitol free cigarettes.
COGGINS *et al.* (242) studied the influence of added sorbitol on the toxicity of cigarette mainstream smoke in *in vitro* assays. Compared to the mainstream smoke of additive free control cigarettes both cytotoxicity and mutagenicity were not affected by any level of added sorbitol. Moreover, the exposure of rats of both sexes in a 90-day nose-only inhalation study to mainstream smoke of cigarettes containing sorbitol showed some minimal effects in the respiratory tract compared to animals exposed to smoke from sorbitol free control cigarettes. None of the observed effects were consistent for either sex and no evidence of a dose-response relationship was seen.
Therefore, it is unlikely that the addition of sorbitol to cigarette tobacco enhances the health risks of smoking

4.5.5. Sugars

- Use and toxicological assessment

Sugars, such as glucose, fructose and sucrose, are natural components of tobacco, especially of the Oriental and Virginia types (24).

- Inclusion level in cigarettes, transfer and pyrolysis

In manufacturing tobacco products, various sugars and sugar containing additives, such as fruit juices, corn and maple syrup, and honey, are added to tobacco. Pure sugars used as tobacco additives are glucose, fructose, invert sugar and sucrose. The polysaccharide cellulose and cellulose derivatives serve as binders in reconstituted tobacco production. As reconstituted tobacco is a component of nearly all commercial tobacco blends for cigarettes, cellulose must also be regarded as a typical carbohydrate additive (420).
As sugars are generally non-volatile, only minor amounts (less than 0.5%, mainly glucose and fructose) are transferred unchanged into cigarette mainstream smoke (417, 421). In 1959, KOBASHI and SAKAGUCHI (417) identified qualitatively by paper chromatography traces of glucose, fructose, arabinose and xylose in the smoke condensate of cigarettes made from different tobacco types. GAGER *et al.* (421) used for their study in 1971 cigarettes made from Burley tobaccos with added ^{14}C-labeled glucose, fructose and sucrose, resp., and determined the intact sugars in mainstream and sidestream smoke.
In 1957, GILBERT and LINDSAY (422) reported that the pyrolysis in pure nitrogen at a temperature of 650 °C of neat glucose, fructose, sucrose and other tobacco constituents, such as cellulose, starch and pectins, produced polycyclic aromatic hydrocarbons, including benzo[*a*]-pyrene. In line with the reasoning of STOTESBURY *et al.* (196) this finding should not be equated without due consideration to what may happen in a burning cigarette.
In their study of sugar pyrolysis in the 1960s, JOHNSON *et al.* (423) reported the generation from sucrose of low boiling carbonyls, such as methylcyclopentanones, lactones and various oxygen containing heterocyclic carbonyls, like alkylated furfurals. Sucrose was pyrolyzed in a glass flask heated with a gas burner. Also in the 1960s, KATO *et al.* (424) and KATO (425) investigated the pyrolysis products of saccharides, such as cellulose and cellobiose, and showed that acetaldehyde, furfural and furan were the most abundant components in the pyrolysates. The materials were pyrolyzed in helium at three different temperatures (250 °C, 350 °C and 500 °C); the pyrolyzer was directly connected to a gas chromatograph. In view of the experimental conditions, this study provides, at the best, some information on which products may possibly be generated from tobacco carbohydrates during cigarette smoking.
In 1969, FAGERSON (426) reviewed studies on the effects of high temperatures on carbohydrates (about thirty were published between 1912 and 1969). Several low boiling carbonyls, such as aldehydes and ketones, furans and furanones, were reported as pyrolysis products.
In a review published in 1976, ROBERTS *et al.* (427) listed over 140 pyrolysis products generated from carbohydrates. Of these, 80% had been identified also in tobacco smoke. Oxygen containing substance classes, such as aldehydes, ketones, acids, ethers and phenols, were most abundant.
In 2002, SANDERS *et al.* (428) prepared a review of published studies on the pyrolysis chemistry of neat mono-, di- and polysaccharides, with emphasis on D-glucose, D-fructose, sucrose and cellulose. Most of the studies had mechanistic value and were not representative of the processes going on in a burning cigarette. The product profiles of the substances obtained by pyrolysis depended on experimental conditions, particularly on pyrolysis temperature and residence time, atmosphere (reducing or oxidizing) and the presence of other substances, such as acids, bases and salts. At higher temperatures (> 800 °C), polycyclic aromatic hydrocarbons (PAHs) were the major products in the tarry phase of the carbohydrate pyrolysates. At lower temperatures (300–600 °C), the pyrolysis of pure cellulose generally favored the formation of levoglucosan as well as low molecular weight oxygenated products, such as carbonyls, furans, etc. The pyrolysis of D-glucose, D-fructose and sucrose appeared to promote the production of furans rather than anhydro-sugars, such as levoglucosan,

and low molecular weight carbonyl compounds. The presence of acids or bases increased the yields of lower molecular weight oxygenated compounds. Turning to cigarette smoke in general, SANDERS *et al.* stated that tobacco represented a complex matrix and during puffing, there existed a wide range of temperatures and a considerable variation of oxidizing and reducing atmospheres. It is remarkable that for all that pyrolysis studies seem to provide clues for understanding possible precursor-smoke constituent relationships in cigarette mainstream smoke.

- Attractiveness and addictiveness

The attractiveness of sugar containing cigarettes was explained by TALHOUT *et al.* (429) on the grounds of sugars in tobacco products masking the harshness of smoke by generating organic acids. This way, smoking would be more attractive, especially for young people. The scientific basis for this allegation still needs to be identified.
Sugars in tobacco were brought into play as precursors of acetaldehyde in smoke (17, 155, 157, 429). It had been shown that acetaldehyde and nicotine when applied together exhibited synergistic positive reinforcing effects in self-administering rats (152). It was further speculated that, by reacting with certain aromatic amines, acetaldehyde may give rise to β-carbolines - a group of compounds possibly inhibiting the activity of monoamino oxidase (430, 431). With these notions in mind it was assumed that acetaldehyde generated from sugars might enhance nicotine addictiveness (92, 429). This is discussed in detail in Section 3.3 of this review on page 421–422.

- Effect on cigarette mainstream smoke composition

DE LA BURDE *et al.* (432, 433) took an interesting experimental approach involving tobacco. They used ^{14}C uniformly labeled glucose and fructose to examine the transformation of carbohydrates during the thermal treatment of flue cured tobacco up to 60 °C for 1–9 days. The levels of reducing sugars in tobacco decreased while the content of free acids, such as acetic acid, increased. Simultaneously, a small amount of carbon dioxide was liberated and an equimolar amount of oxygen consumed from the atmosphere. Between 1.7% and 3.6% of the radioactive glucose was transformed into ether soluble components. Two of them were identified as furfural and hydroxymethyl furfural. This experiment cannot be regarded as a typical pyrolysis study; much rather, it is a trial on the effects of flue curing on tobacco sugars.
Backed by exploratory pyrolysis studies with neat carbohydrates as well as flue cured and Burley tobacco, BELL *et al.* (434) investigated the contribution of glucose to the yields of phenol and alkylphenols in mainstream and sidestream smoke. Cigarettes made from tobacco spiked with uniformly labeled ^{14}C-glucose were smoked in a specially designed apparatus and the fate of the label was followed. Based on sophisticated estimates and calculations the authors inferred that the carbohydrates of tobacco were important precursors of phenol in cigarette smoke but could not alone explain the total phenol yield from tobacco.
A study performed in 1969 by KABURAKI *et al.* (173) with cigarettes made from domestic Japanese tobacco blended with cellulose showed that mainstream smoke contained more than twice the amount of acetaldehyde compared to a control cigarette without added cellulose. In addition, the authors showed that the major components in the vapor phase of the smoke were produced from the skeletal substances of tobacco like cellulose. It was also confirmed that most of 2-methylfuran and 2,5-dimethylfuran, characteristic constituents of the vapor phase of smoke of flue cured tobaccos, was produced from glucose, fructose or sucrose in the tobacco leaves.
In 1970, BEST (435) examined the effects of mono- and disaccharides, which were either inherent in or added to flue cured tobacco of various qualities. This study has been available to the public since 1998. The differences in the organic acid levels found by BEST were assumed to be caused by the distinctive sugar content of the tobaccos tested.
THORNTON and MASSEY (436) investigated the effects of glucose and fructose on the carbonyl and acid yields of mainstream smoke when added in different concentrations (10.5–17.8%) to a series of cigarettes made from Burley tobacco. Compared to controls (no reducing sugars added) there was virtually no change in the yields of volatile aliphatic aldehydes and other aliphatic carbonyl compounds. However, an increase in the formation of 2-furfural was observed, especially when fructose was added. The conversion rate of fructose to 2-furfural was only 1–2%.
PASSEY and ELSON (437) claimed that sugar containing tobaccos generated more acidic cigarette mainstream smoke.
In 1975, PHILLPOTTS *et al.* (162) reported the total aldehyde levels in the mainstream smoke of 42 major United Kingdom cigarette brands with a total tobacco sugar content between 14.3% and 19.7%. In addition, cigarettes from the markets of other European countries were studied. In the U.K. brands, no relationship between the sugars in tobacco and total aldehyde yields in mainstream smoke could be shown. When cigarette data from France, West Germany, Belgium, Holland, Italy, Luxembourg, Switzerland and Norway were evaluated, there was - at first sight - a relationship between mainstream smoke aldehyde yields and tobacco sugars. However, closer inspection of the data showed that Italian brands had low sugar and low aldehyde yields, while "French" cigarette brands had even lower sugar levels but higher aldehyde yields. A significant correlation was found between mainstream "tar" yields and total aldehydes. These findings were valid also for the most abundant aldehyde in mainstream smoke, acetaldehyde.
It should be mentioned that in 1975 nearly all U.K. cigarette brands were made without additives from flue cured tobaccos, which, however, were rich in natural sugars. Most of the cigarette brands on the French and Italian markets contained additive free dark, air cured tobaccos, low in sugars, while in West Germany and other central European countries cased and flavored American blend cigarettes with added sugars were predominant. The influence of tobacco (bright flue cured, dark air cured, American blend) - but not of sugars - on cigarette mainstream smoke total aldehyde and, specifically, acetaldehyde yields was clearly visible in the study data.
The results of PHILLPOTTS *et al.* (162) were confirmed in two subsequent publications. SEEMAN *et al.* (167) con-

cluded in a review of the scientific literature that sugars, such as D-glucose, D-fructose and sucrose, did not produce greater yields of acetaldehyde in cigarette mainstream smoke than tobacco on a weight-by-weight basis. The natural tobacco polysaccharides, including cellulose, were assumed to be the primary precursor of acetaldehyde in mainstream smoke. In addition, the review addressed the bioavailability of mainstream smoke acetaldehyde. Its deposition and uptake in the smoker's upper respiratory tract, including the mouth, is followed rapidly by metabolic conversion by aldehyde dehydrogenase rendering very unlikely any direct effects on the central nervous system of the smoker.

The evaluation of an industry database by SEEMAN *et al.* (174) revealed that the mainstream smoke acetaldehyde levels of a large number of commercial U.S. cigarettes correlated significantly with mainstream "tar" and carbon monoxide yields, but not with reducing sugar concentrations in the tobacco blends. Cigarette design characteristics primarily controlled mainstream "tar" and carbon monoxide. Hence, strong correlations between mainstream smoke acetaldehyde and "tar" are most directly explained by variations in the design characteristics of commercial cigarettes and by the kind of tobacco used for cigarette manufacturing.

A review on sugars as tobacco additives and their effect on cigarette mainstream smoke composition was published by TALHOUT *et al.* (429) in 2006. The authors claimed that sugars promoted tobacco smoking because they generated acids that neutralize the harsh taste and throat impact of tobacco smoke. They also speculated that the sweet taste and the pleasant smell of caramelized sugar flavors were appreciated in particular by adolescent novice smokers. In addition, sugars in tobacco were alleged to generate carbonyls, such as acetaldehyde, during smoking. It is disturbing that TALHOUT *et al.* paid no attention to the review of SEEMAN *et al.* (167), published already in 2002, which showed that cellulose rather than sugars was the primary precursor of acetaldehyde in cigarette mainstream smoke. The findings of DENOBLE and MELE (152) and BELLUZI *et al.* (171) that acetaldehyde had addictive properties and acted synergistically with nicotine as an addiction enhancing agent in rodents were also quoted without any comment or critique. It was pointed out that many toxic smoke components (some of them carcinogenic) were generated from sugars by pyrolysis. In particular, sugars increased the levels of formaldehyde, acetaldehyde, acetone, acrolein and 2-furfural in tobacco smoke. No attention was paid to the results of various *in vitro* and *in vivo* bioassays, which examined the effects of sugars on smoke toxicity. TALHOUT *et al.* (429) concluded that sugars in tobacco contributed significantly to the adverse health effects of tobacco smoking and the use of sugars in cigarette manufacturing should be restricted by law. As the type of curing largely determines the final sugar level of tobacco products, the impact of such methods should also be taken into account when regulatory measures on sugars were considered.

In a letter to the Editor, BAKER (181) responded in 2007 to the conclusions and assertions of TALHOUT *et al.* (429). He agreed with TALHOUT *et al.* that sugars in tobaccos increased the levels of formaldehyde and there was some evidence that sugars increased the levels of 2-furfural in mainstream cigarette smoke. However, he disagreed with the conclusions that the yields of acetaldehyde and acrolein were increased by sugars, that sugars contributed to the adverse health effects of tobacco smoking, and that the allegedly sweet taste by adding sugars to tobacco was particularly appreciated by starting adolescent smokers. Similarly, scientific data concerning these effects of sugars when used as tobacco additives, published in a number of peer reviewed journals, did not support the conclusions and assertions of TALHOUT *et al.* BAKER stated with respect to his own studies: "*I believe that our conclusions are valid ones and that there is a wealth of solid evidence to substantiate them, obtained by a variety of studies over many years*."

Recently, CAHOURS *et al.* (179, 180) performed a re-analysis of the data on sugars in tobacco and cigarette mainstream smoke acetaldehyde yields from the 83 European commercial cigarette brands studied in the 1970s by PHILLPOTS *et al.* (162) and more recent industry data for a range of 97 European commercial cigarettes (analyzed in 2001–2010) containing natural sugars or inherent plus added sugars. These cigarettes included brands made from American blends, Virginia tobaccos and dark air cured tobaccos. The analysis produced also data for 65 specifically prepared experimental cigarettes made from single curing grades of tobacco (16 sun cured, 29 air cured and 20 Virginia). Air cured grades had a sugar content of 0–0.3%, sun cured grades of 3.6–15.3%, and flue cured Virginia grades of 1.3–23.7%. It was shown in this extensive study that there was no relationship between sugar in the blend and acetaldehyde yields even when multivariate analysis was carried out, which took cigarette mainstream smoke nicotine free dry particulate matter (NFDPM) into account as a co-factor. In this kind of re-analysis each known factor contributing to mainstream smoke acetaldehyde yields must be considered in order to avoid misleading conclusions.

The data set used by PHILLPOTS *et al.* (162) had previously been re-examined and interpreted by O'CONNOR and HURLEY in 2008 (438) using multivariate analysis. They had concluded that sugars in tobacco blends accounted for an additional 11% variance in aldehydes. However, CAHOURS *et al.* (180) argued that the multivariate analysis approach used by O'CONNOR and HURLEY was incomplete and had generated misleading conclusions.

Furthermore, CAHOURS *et al.* (180) recognized no difference between the mainstream smoke acetaldehyde yields of cigarettes with American blends, dark air cured or flue cured tobaccos, irrespective of their sugar content when NFDPM yields were taken into account. Nor were differences seen in the mainstream smoke acetaldehyde yields of the 65 experimental cigarettes made from single grades of either flue cured Virginia, sun cured or air cured tobaccos - all with no sugar added.

The study of CAHOURS *et al.* (180) supports the assumption of SEEMAN *et al.* (167, 174) that structural tobacco materials (such as celluloses) are the main source of acetaldehyde in mainstream cigarette smoke.

In 2006, BAKER (439) published an overview on the generation of formaldehyde in cigarette smoke. Yields of formaldehyde in mainstream smoke were reported to be in the range of 1.3 μg in filter cigarettes to 283 μg in unfil-

tered cigarettes. By comparison, 60 µg is a more typical upper limit for modern filter cigarettes (67, 440, 441) smoked under ISO standard smoking conditions (73). Using four cigarette prototypes with sugars added to tobacco (glucose, fructose, sucrose and mixtures of the three) BAKER (439) found elevated yields of formaldehyde in mainstream smoke in all cases when smoked under ISO standard conditions (73). Increases up to 60% were observed with maximum sugar levels (7% invert sugar). Machine smoking of the test cigarettes under more intensive regimens (puff volume and/or puff frequency increased) advanced mainstream formaldehyde yields parallel to the amounts of added sugars. Different sugars increased formaldehyde yields to different degrees. The highest increase was observed with invert sugar, the lowest with brown sugar. BAKER also demonstrated that the presence of ammonium compounds and amino acids in tobacco inhibited partly the generation of formaldehyde from sugars. For instance, sugar materials, such as honey and maple syrup, contain amino compounds, which influence formaldehyde generation.

BAKER (439) pointed out that the addition of various sugars to tobacco had some statistically significant effects on the yields of other "Hoffmann" carbonyl mainstream smoke constituents, such as acrolein, crotonaldehyde, acetaldehyde, propionaldehyde, n-butyraldehyde and acetone, analyzed concurrently with formaldehyde. The effects were generally small (under 16%) and not consistent amongst the different series of test cigarettes. Contrary to mainstream smoke, the sidestream smoke yields of formaldehyde were not affected by sugar addition to cigarette tobacco.

The effect of sucrose added to a cigarette blend on the generation of formaldehyde was confirmed in the tobacco additives study initiated by the German regulatory authorities (176–178).

The experimental studies of THORNTON and MASSEY (436) and PHILLPOTTS *et al.* (162), the reviews of SEEMAN (167, 174), the review and study of BAKER (439) and the investigation initiated by the German regulatory authorities (176–178) do not confirm the conclusion of TALHOUT *et al.* (429) that tobacco sugars (natural or added) are important for the carbonyl yields of cigarette mainstream smoke, especially for acectaldehyde, and contribute to the addictiveness of cigarette smoking and the attractiveness of tobacco consumption for adolescents. As reported by SEEMAN *et al.* (167, 174), BAKER *et al.* (208) and BAKER (439) carbonyls in mainstream smoke - with the exception of formaldehyde - correlated significantly with mainstream "tar" and the amount of tobacco burned during puffing but not with sugars or sugar containing additives.

COGGINS *et al.* (242) analyzed the composition of mainstream cigarette smoke following the addition of carbohydrates and sugar containing natural products to tobacco. Experimental cigarettes were produced containing the additives, β-cyclodextrin, cleargum (sodium starch octenyl succinate), sorbitol, high fructose corn syrup, honey, invert sugar, maltodextrin, molasse, plum juice concentrate, raisin juice concentrate and sucrose. Two or three different levels of each additive were used. Compared to the smoke of additive free cigarettes the additives produced generally only minimal changes in smoke chemistry and consistently a small increase in formaldehyde. With D-sorbitol and sucrose significant reductions were observed for some smoke constituents. This may in part have been an effect of the replacement of tobacco by the additive in the experimental cigarettes.

There are two particular studies, which address the relationship between tobacco sugars and carbonyls in smoke but had quite different original objectives.

The goal of a 1982 Canadian study by ZILKEY *et al.* (442) was "*to determine, for Canadian-grown tobaccos, the effects of various bright and burley tobacco blends, with and without incorporated tobacco sheet or a tobacco substitute or high efficiency filtration, on certain chemical and physical properties of tobacco and tobacco smoke*". The cigarettes used differed in weight (0.87–1.23 g), tobacco nicotine (0–2.36%), reducing sugars in tobacco (0–20.4%), puff number (5.01–10.48), "tar" level (4.14–26.04 mg/cig) and mainstream smoke total carbonyl yield (303–1,292 µg/cig). The reason for these differences was primarily the composition of the blends. Cigarettes made from the tobacco and nicotine free tobacco substitute, Cytrel® (produced from modified cellulose), and from different kinds of reconstituted tobaccos blended with the tobacco substitute and/or different grades of Bright tobacco were certainly not suitable for evaluating the relationship between reducing sugars in tobacco and carbonyls in cigarette mainstream smoke. It must be assumed that the cigarettes in this study obscured a possible relationship between sugars and carbonyls due to the composition of the tobacco substitute, Cytrel®, and the different tobacco sheets. Consequently, well-founded conclusions seem impossible.

The other investigation is a 1992 unpublished nicotine/sugar study by SHELAR *et al.* (443). They reported that the amount of sugars (4–16%) added to two different Burley tobacco grades, K1 and K2, significantly decreased the mainstream smoke pH of experimental cigarettes while the type of sugar (glucose, fructose, sucrose) had no effect. It was also stated that total mainstream smoke carbonyls went up as the sugar levels increased, again irrespective of the type of sugar used. (K1 and K2 are two different qualities of U.S. Burley tobacco. K1 indicates low-mid stalk position and K2 mid-upper stalk position. The nicotine content in K1 is lower than in K2.)

The objective of the study of SHELAR *et al.* was "*to determine the type and amount of casing sugar needed on burley tobacco for different nicotine levels and identify a sugar to nicotine ratio that can be used to develop smoother products*". Smoke taste and aroma of the test cigarettes were examined and not the relationship between sugar levels in tobacco and their effect on carbonyl yields in mainstream smoke. The test cigarettes used in this study were manufactured at constant firmness. The amount of sugar applied on tobacco influenced the filling power of the cut tobacco. Therefore, the tobacco weight of the cigarettes differed. Unfortunately, the authors gave no precise description of the test cigarettes. However, the variation in puff numbers (K1 cigarettes: 4.6–7.1; K2 cigarettes: 5.8–8.3), in "tar" values (K1: 10.5–13.4 mg/cig; K2: 14.6–16.6 mg/cig) and smoke nicotine (K1: 1.05–1.53 mg/cig; K2: 1.95–2.68 mg/cig) showed clearly that these cigarettes were not homogeneous and, therefore, not suitable for a good analytical study, in particular the evaluation of the relationship between sugars in tobacco and carbonyl yields in mainstream smoke.

A comprehensive review on sugars used as components of casing materials for cigarette tobaccos was published by RODGMAN (21); see Section 4.3. on page 429.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

Sugars are natural tobacco constituents. Therefore, it is not easy to evaluate whether, or to which extent, the addition of sugars to tobacco increases the overall toxicity of cigarette mainstream smoke.

A reduction of the mutagenicity of cigarette smoke condensate in the Ames assay was observed when the sugar level in tobacco was increased (256, 419). Glucose, fructose, galactose, sucrose and lactose were effective, with fructose producing the greatest reduction in mutagenicity.

Already in 1963, WYNDER and HOFFMANN (254) had shown that the mainstream smoke condensate of cigarettes made from tobaccos high in sugars (flue cured Virginia or Oriental) exhibited a higher specific tumorigenicity on mouse skin than smoke condensate from low-sugar tobaccos (Burley, Maryland). Interestingly, higher acidity of cigarette smoke condensate did not change the specific tumorigenicity (13).

In the NATIONAL CANCER INSTITUTE (NCI) program "Towards a less hazardous cigarette" (68) the effects of added invert sugar on cigarette smoke chemistry and toxicity were investigated. Smoke condensate of cigarettes containing invert sugar painted on mouse skin had no increased specific tumorigenicity at a dose of 12.5 mg/day compared to condensate from cigarettes without invert sugars; however, an increase was seen with 25 mg/day as stated in Report No. 5 of the NCI program (444). It must be taken into account that besides invert sugar glycerol was added to the blend of these experimental cigarettes. The addition of invert sugar alone or glycerol alone to the blend produced no change in the specific tumorigenicity of the smoke condensate.

The contrasting results obtained with cigarette smoke condensate from sugar containing cigarettes (a possible increase of the specific tumorigenicity in the mouse skin assay *vs.* a decrease of the mutagenicity in Ames bacterial tests) are an indication of the limitations when evaluating the biological properties of cigarette smoke. Ageing and the possibility of artifact formation during condensate preparation as well as the exclusion of the smoke gas phase may influence the test results (445).

R.J. Reynolds Tobacco Co. developed a test strategy to evaluate the toxic potential of (new) additives. Honey, used as a casing ingredient, is a pertinent example (446). The mainstream smoke properties of test cigarettes made from a standard commercial tobacco blend, with the Burley tobacco containing 5% honey, were compared to a control cigarette containing 5% invert sugar instead of honey. Principal mainstream smoke yields (nicotine, "tar", carbon monoxide, low molecular aldehydes and ketones, phenols, ammonia, furfurals, nitrogen oxides, hydrogen cyanide and benzo[*a*]pyrene) were analyzed. Ames tests as well as sister chromatid exchange assays with Chinese hamster ovary cells were performed. The effects of smoke condensate were evaluated in a SENCAR mouse skin painting assay regarding tumor promotion. Sprague-Dawley rats were used in a sub-chronic 13-week nose-only mainstream smoke inhalation study. The results of the investigation demonstrated that honey instead of invert sugar as casing material did not alter the overall toxicity of cigarette mainstream smoke.

Using a comparable test strategy the toxicity of high fructose corn syrup was evaluated (447). The effects of this casing material on mainstream cigarette smoke were compared to corn syrup/invert sugar (control). Collectively, the data for mainstream smoke chemistry, genotoxicity, dermal tumor promotion in mice and sub-chronic inhalation toxicity in rats demonstrated no differences between test and control cigarettes.

COGGINS *et al.* (242) observed in a study (already mentioned above) of the influence of carbohydrates and carbohydrate containing preparations, when used as tobacco additives, on the toxicity of mainstream smoke that cytotoxicity and mutagenicity were essentially the same for all experimental cigarettes tested. Individual sub-chronic 90-day smoke inhalation studies in rats with 10 of the 11 additives showed very few statistically significant differences that were largely sporadic and inconsistent between sexes. In no case was there a statistically significant dose relationship between inclusion level and increased severity score, even at high inclusion levels compared to the levels used in commercial cigarettes.

Recently, ROEMER *et al.* (448) published a review of scientific studies on the use of sugars as tobacco additives. The reasons for the addition of sugars in manufacturing, biological data related to sugars transferred unchanged from tobacco into mainstream smoke, and their fate during smoking and influence on smoke composition and toxicity were discussed. The review included information on smoke exposure and smoking behavior comparing markets of American blend cigarettes with additives, including sugars, to additive free Virginia cigarettes. The comparison of American blend and Virginia cigarette markets regarding smoking related lung cancer and chronic obstructive lung disease undertaken by LEE *et al.* (238) was also considered. Evaluating mainstream smoke chemistry data in relation to smoke nicotine of cigarettes with and without added sugars, a simulation of the differential smoking related exposures to these constituents was performed, and statistically significant quantitative changes were identified. According to the authors, this approach offered the most discriminatory analysis of potential changes in mainstream smoke exposure resulting from the use of a particular additive in a research cigarette. ROEMER *et al.* summarized the outcome of their assessment: "*While some changes with sugar application were detected, the overall evaluation of all data considered on a weight-of-evidence basis suggests that the use of sugars would add no significant toxicity to tobacco products and therefore could be considered safe in the context of this use. This conclusion is based on the results of chemical analytical, in vitro, and subchronic inhalation studies with research cigarettes with and without sugars as tobacco ingredients.*" Comparing smoking behavior and smoke uptake in smokers of American blend and Virginia cigarettes "*... for nicotine uptake levels, no indication of sugar application-related differences could be derived. The data analyzed do not support*

concerns that the use of sugars as ingredients would increase tobacco smoking dependence. No difference in mortality due to smoking-related diseases could be detected between American-blend and Virginia-type markets ...". The review of ROEMER *et al.* (448) is an instructive synopsis of the effects of the additive "sugar" on cigarette mainstream smoke composition and toxicity, smoking behavior, nicotine uptake and mortality from smoking-related lung cancer and chronic obstructive pulmonary disease. However, in our opinion, the comparison of smoking behavior, nicotine uptake and the risk for smoking related diseases between American blend and Virginia cigarette markets is above all an indication of other cigarette additives besides sugars not influencing these parameters because the sum of sugars in the tobacco of American blend and Virginia cigarettes is of quite comparable magnitude.

In summary, it can be concluded from the scientific evidence presented above that sugars and the kind of sugar used as additive have - if any - only small and unimpressive effects on cigarette mainstream smoke toxicity.

4.5.6. Cocoa

- Use and toxicological assessment

Cocoa, cocoa extract and chocolate are widely used as casing components for American blends and intended to enhance natural Burley taste (48). Cocoa is part of the casing formulation of nearly all commercial American blend cigarettes. The complex composition of cocoa was described by HARLLEE and LEFFINGWELL (449) in 1978. About 60% of the volatile substances identified in cocoa are also components of tobacco and/or tobacco smoke. Cocoa contains numerous pyrazines and the pharmacologically active methylxanthines, theobromine and to a lesser amount caffeine. This means for the smoke that the tobacco additive "cocoa" contributes to the total content of pyrazines and adds theobromine and caffeine.

Theobromine and caffeine are present in cocoa at levels of about 2.6% and 0.2%, resp. (450, 451). There were increasing concerns regarding the safety of the methylxanthines in the human diet and luxury foods, especially regarding their potential carcinogenicity, and reproductive and developmental toxicity.

Oral LD_{50} values for theobromine in animals were found to be about 1 g/kg body weight or higher (452). Using a series of *in vitro* assays BRUSICK *et al.* (453) saw no effect of theobromine in the Ames assay, the chromosome aberration assay with CHO cells and the transformation assay with Balb/c-3T3 cells. However, biological activity was observed in the mouse lymphoma assay and the sister chromatid exchange assays with human lymphocytes and CHO cells. Such mixed results are difficult to interpret. ROSENKRANZ and ENNEVER (454) analyzed the data reported by BRUSICK *et al.* (453) by the Carcinogen Prediction and Battery Selection (CPBS) method (455) - a procedure that can be used to predict potential carcinogenicity on the basis of the results of short-term tests. For theobromine, the analysis did not predict a potential for causing cancer by virtue of a genotoxic mechanism.

TARKA *et al.* (450) evaluated the chronic toxicity and carcinogenicity of cocoa powder. The powder was fed at three levels (1.5%, 3.5% and 5.0% in the diet) for 104 weeks to male and female Sprague-Dawley rats. Compared to control, survival rates of the treated animals were similar, and no evidence of treatment related diseases and effects was noted. Although there was no significant difference in the incidence of benign mammary gland fibroadenomas in female rats between any cocoa powder fed group and the control group, a marginally significant trend to develop these fibroadenomas was apparent. The authors considered the significance of the finding doubtful as the incidence of this lesion in the highest dose group was well within the range seen in historical control groups of this rat strain. No evidence of carcinogenicity from dietary cocoa powder was found in either sex.

Teratogenicity studies of cocoa powder and theobromine in rats and rabbits showed no significant effects other than delayed ossification of the sternebrae at doses, which approached maternally toxic levels (456, 457). In a three-generation reproductive study of cocoa powder in rats, a marginal increase of the incidence of testicular atrophy was observed but this effect failed to reach statistical significance. Reproductive indices were found to be unaffected by 5% cocoa powder in the diet (458).

- Inclusion level in cigarettes, transfer and pyrolysis

Theobromine, being a characteristic constituent, may be used for the determination of cocoa in tobacco products by spectrophotometric (459, 460) and gas chromatographic (460) methods. The cocoa concentration in the tobacco of American blend cigarettes is about 2% or less, corresponding to 0.05% of theobromine or less. In a spiking study with 0.1% and 0.2% theobromine on cigarette tobacco a 13% transfer rate into mainstream smoke was determined (460).

In 1978, SCHLOTZHAUER (461) studied the pyrolysis of cocoa powder at different temperatures (350–750 °C) in a nitrogen atmosphere. He concluded that cocoa powder as tobacco additive would not significantly enhance the phenol and catechol content of tobacco smoke. PARK *et al.* (462) concluded from their pyrolysis studies in a nitrogen atmosphere that the major pyrolysis products of cocoa were aliphatic hydrocarbons and phenolic compounds. However, the conditions used in both studies (461, 462) were far from the reality in a burning cigarette.

The proteins of cocoa contain about 1.5% tryptophan and 19% glutamic acid (21). It was shown that these amino acids may generate carcinogenic *N*-heterocyclic amines when pyrolyzed (463, 464). According to RODGMAN (21), none of these heterocyclic amines, however, were detected in the pyrolysate of cocoa or in the smoke of cocoa treated tobacco.

- Attractiveness and addictiveness

The addition of cocoa and cocoa containing materials at normal use levels of 1–2% (48) does not result in a sweet and/or chocolate like taste of the smoke. Cocoa contains theobromine and to a lesser amount its homologue, caffeine. Theobromine acts as a bronchodilator (465). Therefore, it was speculated that the addition of cocoa to cigarette tobacco facilitated inhalation and, consequently, the uptake of nicotine (17).

The therapeutic principle of bronchodilation with theobromine calls for sustained plasma levels of approximately 10–20 mg/L. For reaching this level, the administration of about 450 mg theobromine during a day was necessary resulting in a peak plasma level of 9.8 mg/L, as shown in a clinical study by SIMONS *et al.* (465). Because of its relatively weak pharmacological effects theobromine is no longer used for therapeutic purposes.

In a pharmacokinetic model, with 40 cigarettes with 5% cocoa smoked during the day, assuming 100% absorption of theobromine by the lungs, 5.2 mg theobromine are taken up by the smoker. Under these conditions the peak plasma level of theobromine was estimated to be 0.08 mg/L, equal to only 1/125–1/250 of the therapeutically effective concentration (146). Therefore, it can be ruled out that the addition of cocoa to cigarettes results in bronchodilating effects during smoking and facilitates the uptake of nicotine.

In 2003, RAMBALI *et al.* (466) evaluated the contribution of cocoa to cigarette addiction. This report was compiled for the Directorate for Public Health of the Ministry of Health, Welfare and Sports and the Inspectorate for Health Protection and Veterinary Public Health of the Netherlands, within the framework of the project "Reduction of health and addiction risks of smokers". The ten best known psychoactive cocoa compounds (theobromine, caffeine, serotonine, histamine, tryptophan, tryptamine, tyramine, phenylethylamine, octopamine and anandamine) were included in the evaluation. The authors concluded that systemic effects of the psychoactive constituents of cocoa via cigarette smoking seemed unlikely, also because the psychoactive biogenic amines present in cocoa are degraded rapidly. Increased nicotine absorption due to effects of theobromine and caffeine on bronchodilation or of histamine on bronchoconstriction was considered improbable because the levels of these compounds in cigarette smoke were found to be too low for exerting any local bronchoactive effects. The authors' general conclusion was that "*the level of these compounds in added cocoa in cigarettes is not sufficient to increase the addiction to cigarette smoking*".

- Effect on cigarette mainstream smoke composition

The effects of cocoa on cigarette mainstream smoke composition were described in the U.S. NATIONAL CANCER INSTITUTE Report No. 3 "Towards a Less Hazardous Cigarette" (68). In a study, 1% cocoa powder was added to the Standard Experimental Blend III (SEB III). When smoking test cigarettes a minimal increase of the phenol and PAH yields in mainstream smoke was observed compared to the smoke of cocoa free control cigarettes, together with a larger increase of the yields of catechol and total fatty acids.

RODGMAN (21) reviewed the effects of cocoa on cigarette mainstream smoke. Special emphasis was put on the contribution of cocoa to the levels of polycyclic aromatic hydrocarbons (PAHs) in the smoke (see Section 4.3. on page 429).

The effect of a number of cocoa materials, when used in combination with various other casing and flavoring additives, was examined by RUSTEMEIER *et al.* (230). Test cigarettes contained food type cocoa (0.65% and 0.97%), cocoa extract (up to 772 ppm), cocoa shells (0.99% and 1.48%) or cocoa shell extract (up to 0.19%) (229). TPM yields were increased with all test cigarettes. When normalized to TPM, the majority of the 51 smoke constituents analyzed were reduced compared to control while an increase was observed for a few. Due to the presence of a multitude of additives in the test cigarettes it is difficult to suggest specific causal relationships.

BAKER *et al.* (208) confirmed in their study on casing ingredients and smoke chemistry that there was no significant influence of cocoa on mainstream smoke PAH yields, especially benzo[*a*]pyrene.

In the study initiated by German regulatory authorities (176–178) the influence of cocoa powder in cigarettes on mainstream smoke composition was examined. A pronounced decrease of the levels of tobacco specific *N*-nitrosamines was observed by HAHN and SCHAUB (176) and INTORP *et al.* (178) following the addition of cocoa powder. ROEMER *et al.* (177) reported that cocoa powder in test cigarettes did not result in any consistent effects on the mainstream smoke analytes measured.

COGGINS *et al.* (244) examined the effects of five different cocoa-derived additives (chocolate, two batches of cocoa, cocoa-grand prix black, cocoa nibs tincture and cocoa shell extract) on mainstream smoke composition and toxicity. Each material was added to tobacco at three different levels; the characteristic component, theobromine, was used for assessing the levels of the cocoa-derived additives in the experimental cigarettes (up to around 40,000 ppm). A broad range of smoke constituents was analyzed, including "tar", nicotine and CO, aliphatic and aromatic hydrocarbons, aldehydes, phenols and amino compounds, NNN and NNK and several polycylic aromatic hydrocarbons.

No consistent changes were found in the analytical chemistry results. There were several instances, in which the level of a particular smoke constituent differed significantly between experimental and control cigarettes but these observations were sporadic and only rarely dose dependent. The independent analytical chemistry studies with two batches of cocoa cast a light on the degree of variability associated with cigarette manufacturing and the analysis of particular mainstream smoke constituents. These factors are very important for the fair assessment of the potential effects of tobacco additives and have been discussed in great detail by GAWORSKI *et al.* (251).

In summary, the tobacco additives, cocoa and cocoa preparations, showed no impressive and relevant effects on cigarette mainstream smoke composition.

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

ROEMER *et al.* (231) evaluated the effects of mainstream smoke on *in vitro* cytotoxicity and genotoxicity with cigarettes containing mixtures of additives including cocoa and cocoa shells (and their extracts). No significant differences were observed between the smoke of cigarettes with additives and additive free cigarettes.

In the ingredients study initiated by the German regulatory authorities, the effect of cocoa powder on mainstream smoke was evaluated (176). ROEMER *et al.* (177) used these

test cigarettes to determine their *in vitro* toxicity. The cytotoxicity of total particulate matter, as measured in the neutral red uptake assay, was not affected by the addition of cocoa. The cytotoxicity of the vapor phase was decreased by 10–15%. As this decrease is lower than the discriminatory power of the assay, it can only be taken as an indication for a reduction of cytotoxicity. The *in vitro* mutagenicity of total particulate matter in the Ames assay was not notably affected by the addition of cocoa in any of the *Salmonella typhimurium* strains with and without metabolic activation.

The Tobacco Working Group of the U.S. NATIONAL CANCER INSTITUTE (NCI) included in their studies a cigarette containing 1% cocoa powder (68). Mainstream smoke condensate of the cigarette was compared to a cocoa free control cigarette in the mouse skin painting assay at two doses (75 mg and 150 mg condensate per week). There was a 5% higher incidence of tumor bearing mice at the low dose and a 20% higher incidence at the high dose in comparison to the control cigarette (significant only at the high condensate dose). The NCI came to the conclusion that the addition of cocoa powder "*appears to increase the tumorigenicity*" in cigarette mainstream smoke condensate.

In 1990, ROEMER and HACKENBERG (467) re-examined the results obtained by the NCI with an extended protocol. In a mouse skin painting bioassay they compared the mainstream smoke condensates of cigarettes with 1.0% and 3.0% cocoa powder added to the filler to cocoa free cigarettes. These cigarettes were similar to those used in the NCI study (68) except the composition of the reconstituted tobacco in the blend. Three condensate doses, 60, 90 and 125 mg, were applied weekly for a 75-week period. All mice alive at the end of the application period were sacrificed. No increase in tumor incidence was seen in the groups with the cocoa containing cigarettes. In particular, for the middle (90 mg) and high (125 mg) condensate doses the incidence of tumor bearing mice was lower for the cigarettes with 1% or 3% cocoa than for the control cigarette. The results of the NCI study (68) were not confirmed. Therefore, ROEMER and HACKENBERG (467) concluded that the NCI finding was probably a chance result and there was no evidence indicating an enhancement of the biological activity of cigarette mainstream smoke condensates derived from cigarettes with up to 3.0% cocoa in the blend.

The results obtained by ROEMER and HACKENBERG were confirmed by the study of GAWORSKI *et al.* (358), in which cocoa was added at levels up to 9.7% to test cigarettes as a component of an additive mixture. There was no increase in the dermal tumorigenicity of cigarette mainstream smoke condensate in mice compared to the condensate from cocoa free cigarettes.

VANSCHEEUWIJCK *et al.* (232) exposed male and female Sprague-Dawley rats nose-only for 90 days and 6 hours each day to air containing 150 µg total TPM/liter from cigarettes containing mixtures of flavorants and casing materials, including cocoa and cocoa shells (and their extracts). No relevant toxicological differences were observed for a broad range of *in vivo* endpoints between the experimental cigarettes with and without additives. Likewise, BAKER *et al.* (239) found no effects of cocoa containing additive mixtures on the toxicity of inhaled mainstream smoke in rats.

Complementing their data on smoke composition, COGGINS *et al.* (244) examined the effects of four different cocoa-derived additives (chocolate, cocoa, cocoa nibs tincture and cocoa shell extract) on mainstream smoke toxicity. Each material was added to tobacco at three different levels; the characteristic component, theobromine, was used for assessing the levels of the cocoa-derived additives in the experimental cigarettes (up to around 40,000 ppm).

Responses in cytotoxicity and mutagenicity tests were unaffected by any of the additives examined. 90-day inhalation studies with rats showed no effects even at high inclusion levels of chocolate and cocoa nibs tincture. A comparable inhalation study with cocoa produced inconsistent results; however, sporadic significant histopathological differences to control were all restricted to one sex and in no case was there a dose-response relationship. The authors concluded that "*even at high inclusion levels there was a lack of toxicological response*" to the cocoa-derived additives under examination.

4.5.7. Licorice

- Use and toxicological assessment

Licorice roots, the base material for licorice, contain about 20% water-soluble extractibles with an appreciable share of glycyrrhizin (typically 3–5% of the root, in some varieties up to 12%) besides mono- and disaccharides (5–15% of the root), starch, gums, flavonoids (1–1.5% of the root) and various other components (44, 45, 46, 468). Glycyrrhizin is a mixture of the potassium and calcium salts of glycyrrhizic acid (synonym: glycyrrhizinic acid).

Licorice extracts may incorporate between 10% and 25% glycyrrhizin, depending on water evaporation (468). The materials used as tobacco additives may contain up to 7% of this compound (46), which is regarded as the primary flavoring agent.

TANAKA *et al.* (469) reported that licorice extract and its component, glycyrrhizin, inhibited in the *Salmonella typhimurium* strains TA 98 and TA 100 the effects of several mutagens, such as 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]-indole (Trp-P-1), which is generated from tryptophan by pyrolysis. The authors considered the inhibition of mutagenicity as proof for the detoxification potential of licorice extracts.

Evaluating the possible health hazards of glycyrrhizic acid in licorice STØRMER *et al.* (470) reported that the high intake of licorice extract may cause hypermineralocorticoidism with sodium retention and potassium loss, edema, increased blood pressure and depression of the renin-angiotensin-aldosteron system. As a consequence, a number of clinical symptoms were observed. Considerable individual variation in the susceptibility to licorice was noticed in humans. In the most sensitive individuals, the regular daily intake of about 100 mg glycyrrhizic acid, corresponding to 50 g licorice sweets, may produce adverse effects. It must be pointed out that an intake of this magnitude, as mentioned by STØRMER *et al.*, cannot be achieved by cigarette smoking or the consumption of other tobacco products. A comprehensive risk and safety assessment regarding the

consumption of licorice root, its extract and powder as a food ingredient was recently published by ISBRUCKER and BURDOCK (471), with emphasis on the pharmacology and toxicology of its active principle, glycyrrhizin. Besides the effects reported by STØRMER *et al.* (470), ISBRUCKER and BURDOCK summarized a number of *in vivo* and clinical studies showing the beneficial effects of licorice consumption including anti-ulcer, anti-viral and hepatoprotective responses. Various genotoxic studies indicated that the active principle of licorice, glycyrrhizin, was neither teratogenic nor mutagenic and may possess anti-genotoxic properties under certain conditions. The pharmacokinetics of glycyrrhizin were described and showed that its bioavailability was reduced when consumed as licorice. This made it difficult to establish clear dose-effect levels in animals and humans. Based on *in vivo* and clinical evidence, ISBRUCKER and BURDOCK proposed an acceptable daily intake of 0.015–0.229 mg glycyrrhizin per kg body weight.

In a blackboard exercise, MASER (472) started from the observation that the activity of four of the five enzymes initiating, and involved in, the metabolism and detoxification processes of the tobacco specific *N*-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), were inhibited by glycyrrhizin and licorice, resp., and speculated that a triple constellation of tobacco-derived carcinogens, enzyme polymorphisms and licorice exposure might potentiate the risk of smokers for lung cancer.

The thoughts of MASER probably have no real relevance for the cancer risk of smoking. The molar mass of glycyrrhizin is around 863. Because of its thermal instability and high molecular weight glycyrrhizin is not expected to be transferred un-decomposed into cigarette mainstream smoke (473). In fact, in a cigarette spiking study no glycyrrhizin was found in mainstream smoke and only a very small amount (under 2%) of a chemically related degradation product, glycyrrhetinic acid (474). Considering further the amounts of glycyrrhizin in cigarettes, which are very low anyway, it can be ruled out that glycyrrhizin is taken up by smoking and capable of influencing the metabolism of the carcinogen, NNK, in humans.

Like theobromine, glycyrrhizin acts pharmacologically as a bronchodilator. Therefore, it was speculated that the addition of licorice facilitated the inhalation of tobacco smoke and nicotine uptake (17). However, the absence of glycyrrhizin in the smoke of cigarettes containing licorice cuts the ground from under this speculation.

Based on a literature survey, VAN ANDEL *et al.* (475) evaluated the health effects and possible addictive effects of licorice preparations when used as a conditioning and flavoring agent in tobacco products. The review centered around the exposure to, and the pharmacology, pharmacokinetics and toxicology of, glycyrrhizic acid and addressed potential addictive aspects. It was concluded that the health risks of adding glycyrrhizic acid in the form of licorice to tobacco seemed small. It was considered unlikely that smoking-related exposure to glycyrrhizic acid would increase mineralocorticoid activity and result in hypertension - effects seen in cases of excessive licorice candy intake. The notion that glycyrrhizic acid acted as a bronchodilator could not be confirmed. No data were available on the dependence potential of glycyrrhizic acid.

- Inclusion level in cigarettes, transfer and pyrolysis

The application level of licorice extract in block, powder or liquid form on cigarette tobacco does not exceed 5% (473) and is generally around 1% (21). The majority of 10 common brands of U.S. cigarettes were found in 1997 to contain 100–300 µg glycyrrhizin per gram of tobacco (117).

In a spiking study by SAKAGAMI (474), glycyrrhizin and its aglycon (and potential degradation product), glycyrrhetic acid (synonym: glycyrrhetinic acid), were added directly to finished cigarettes in very high amounts (up to 10 mg per gram of tobacco). In both cases, only glycyrrhetic acid was detected in mainstream smoke; the transfer rate was found to be remarkably low and hardly exceeded 2%. Glycyrrhizin did not emerge in mainstream smoke undecomposed.

In 1984, VORA and TUORTO (476) communicated the presence of glycyrrhizic acid in the mainstream smoke of cigarettes containing a known amount of licorice. As the report is devoid of all meaningful details - methodologically and quantitatively - its informative value cannot possibly be assessed.

In 1974, GREEN and BEST (477) identified 35 different components in licorice pyrolysates, including phenol and various alkylated phenols and several dimethyl naphthalenes. All 35 compounds were also present in the smoke generated from licorice free tobacco. Subsequently, the same authors (478) determined quantitatively the benzo[*a*]-pyrene content in the pyrolysates of licorice and flue cured tobacco (117 and 133 mg pyrolysate per gram) on a weight-by-weight basis. 24.8 ng benzo[*a*]pyrene per mg was found in the pyrolysate of licorice, 70.5 ng/mg in the pyrolysate of flue cured tobacco.

FRATTINI *et al.* (479) investigated the volatile flavor components of heated licorice essential oil. More than sixty new compounds of various chemical classes were identified reflecting in part the pyrolysis and condensation reactions, which may have occurred at elevated temperatures between the sugars that are so plentiful in licorice extract. The most abundant components were acetol, propionic acid, 2-acetylpyrrole, 2-acetylfuan, furfuryl alcohol, and pyrazine derivatives.

YONGKUAN and WANGYUN (480) studied the pyrolysis of glycyrrhizic acid and its sodium salts. As previously reported by GREEN and BEST (477) several alkylated naphthalenes were detected in the pyrolysate.

CHUNG and ALDRIDGE (481) reported the production of more than 60 components (monocyclic aromatic hydrocarbons, naphthalenes, acids, carbonyls, phenols, esters, ethers, some nitrogen containing compounds, etc.) by the thermal degradation of licorice as the temperature was increased from ambient to 900 °C.

In 2005, CARMINES *et al.* (473) published a comprehensive study on the toxicological evaluation of licorice extract as cigarette ingredient. Three different preparations of licorice available on the market (concentrated syrupy licorice extract, dehydrated block licorice, spray dried licorice powder) were included in the study. The investigation was focused on the pyrolysis of neat licorice preparations, and the influence of these materials on the composition and the *in vitro* and *in vivo* toxicology of mainstream smoke.

Regarding pyrolysis they followed the considerations developed by STOTESBURY *et al.* (196) and discussed earlier in this review. Neat licorice preparations were pyrolyzed to determine potential combustion and pyrolysis products in tobacco smoke. In the pyrolysates, about 60 different components were identified. 1-Hydroxy-2-propanone, phenol and 1,3,6-trimethylnaphthalene appeared to be most prevalent. Minor pyrolysis products were acetaldehyde, formaldehyde and benzene. The major component of licorice extract, glycyrrhizic acid, was not observed in the pyrolysates obtained in this study, indicating that glycyrrhizic acid would not be present in mainstream cigarette smoke.

- Attractiveness and addictiveness

BATES *et al.* (17) speculated on the basis of verbal testimony by the former Philip Morris employee W.A. FARONE in 1997 (482) that licorice was used as tobacco additive for its content of glycyrrhizin. This substance was claimed to act as a bronchodilator facilitating the inhalation of smoke into the lungs and enhancing addictiveness. This speculation was rebutted by MÜLLER and RÖPER (146) using the argument that glycyrrhizin was thermolabile and would not transfer intact to cigarette mainstream smoke in sufficient amounts. Consequently, bronchodilating effects due to smoking licorice containing cigarettes could be ruled out.

- Effect on cigarette mainstream smoke composition

Because of the polycyclic structure of glycyrrhizic acid (a pentacyclic triterpenoid derivative containing two glucuronic acid units; molecular formula: $C_{42}H_{62}O_{16}$; molar mass: 823) it was speculated that polycyclic aromatic hydrocarbons, such as benzo[*a*]chrysene, may be generated during the smoking process (13). This eventuality was thought through - and found to be unwarranted - by RODGMAN (21). RUSTEMEIER *et al.* (230) investigated the effects of licorice extract in combination with other high use level materials (cocoa shells, corn syrup and menthol) on cigarette mainstream smoke composition. The inclusion levels of licorice extract were 1.15% and 1.88% in the test cigarettes. Compared to additive free control cigarettes the test cigarettes produced increased TPM yields by 23% and 16%, resp. Normalized to TPM, an increase of formaldehyde (up to 42%), resorcinol (up to 45%) and lead (13%) was observed. Most of the other components were decreased, e.g., tobacco specific *N*-nitrosamines by up to 37%. This could be explained by the replacement of tobacco by additives in the test cigarettes. The reason for the increase in formaldehyde and resorcinol might be due to the sugar content of the added licorice, that of lead to its presence in the additive. However, as besides licorice other additives were used in manufacturing the test cigarettes, the observed effects could not unambiguously be related to licorice.

It was shown by CARMINES *et al.* (473) in the second part of their investigation that block licorice added as a single additive to cigarette tobacco at a level of 12.5% elevated the amounts of a number of mainstream smoke constituents, including selected polycyclic aromatic hydrocarbons (such as indeno(1,2,3-*cd*)pyrene, benzo[*a*]pyrene and benzo[*a*]anthracene), arsenic, lead, phenol and formaldehyde, while licorice powder at a level of 8% in tobacco did not increase the yields of arsenic and lead. It must be pointed out that the percentage of both forms of licorice was two- to ten-fold the level of licorice commonly used in cigarettes. Application levels typical for cigarettes did not significantly alter the mainstream smoke chemistry profile. Specifically, CARMINES *et al.* showed that glycyrrhizic acid in tobacco below 0.36% produced no statistically significant increase of PAHs - an important observation in light of the fact that typical use levels of licorice extracts result in a glycyrrhizic acid content in tobacco of up to 0.269%.

As mentioned above, elevated levels of arsenic and lead were observed in the mainstream smoke of cigarettes containing 12.5% block licorice. An explanation may be that this batch of block licorice was derived from plant material containing these elements due to normal plant nutrient uptake, the intensity of which depends on soil chemistry.

The increased levels of formaldehyde and phenol in the mainstream smoke of cigarettes particularly rich in licorice (10-fold the typical use level) reported by CARMINES *et al.* are not surprising because licorice extracts may contain considerable amounts of sugars (46). Sugars were reported to contribute to higher formaldehyde when cigarettes are smoked (439) and may also contribute to the increase of phenol levels. A hypothesis to explain the rise of 4-aminobiphenyl levels observed in the mainstream smoke of these cigarettes was not presented by CARMINES *et al.* (473).

- Effect on cigarette mainstream smoke *in vitro* and *in vivo* toxicity

In their study, CARMINES *et al.* (473) investigated the effects of different forms of licorice (block licorice, licorice powder and licorice extract), added in different amounts to cigarettes, on the *in vitro* and *in vivo* toxicity of mainstream smoke. The biological tests indicated no relevant differences in the cytotoxic or mutagenic potential of either mainstream smoke or mainstream smoke condensate of cigarettes with added licorice in any form compared to control cigarettes. In subchronic 90-day nose-only inhalation studies in rats, the mainstream smoke of cigarettes containing 12.5% block licorice caused increased incidence and severity of epithelial hyperplasia in the nose. No relevant respiratory tract changes were seen in rats exposed to the smoke of cigarettes containing 8% licorice powder or extract. In the range of 1.25–5% licorice added (i.e., the level used in cigarettes) no substantial changes in the respiratory tract tissue were observed.

Mineralocorticiod-like effects, said to be associated with excessive licorice ingestion (470, 471), were not found in any of the smoke inhalation studies. The authors (473) concluded from the studies with various forms and amounts of licorice applied to cigarette tobacco that levels up to 5% did not alter the biological effects normally associated with mainstream cigarette smoke. The results of these investigations are in agreement with the work of GAWORSKI *et al.* (358, 362), ROEMER *et al.* (231), VANSCHEEUWIJCK *et al.* (232) and BAKER *et al.* (239).

As discussed earlier, BATES *et al.* (17) speculated that licorice as tobacco additive may cause bronchodilation

facilitating the inhalation of cigarette smoke. In the inhalation studies of CARMINES *et al.* (473) the respiratory physiology of rats exposed to the smoke of cigarettes with licorice up to 10 times the level generally used in cigarette manufacturing was not different from control animals exposed to the smoke of licorice free cigarettes. There was no indication that licorice in tobacco had any effects on tidal volume, respiratory frequency or minute volume. Similar observations were made by VANSCHEEUWIJCK *et al.* (232) in their 90-day inhalation study with rats. CARMINES *et al.* (473) pointed out that there were no biological data to support the contention that in smokers licorice acted as a bronchodilator.
The results of all these studies suggest that the addition of licorice to cigarettes at typical use levels does not lead to an increase in the biological activity of cigarette mainstream smoke as measured by different *in vitro* and *in vivo* assays.

4.5.8. Citric acid

COGGINS *et al.* (248) evaluated the effects of citric acid added to cigarette tobacco on the composition and toxicity of mainstream smoke. The acid was added to tobacco at three different levels (4,400, 17,600 and 44,000 ppm). Sporadic statistically significant differences were observed with regard to the mainstream smoke composition of citric acid containing *vs.* acid free cigarettes but none resulted in significant changes of *in vitro* cytotoxicity or mutagenicity or in the responses measured in a 90-day nose-only inhalation study with rats.

4.5.9. Triacetin

Under ISO standard smoking conditions (73) less than 0.5 mg triacetin is transferred from a cigarette filter into mainstream smoke (483).
The effects of triacetin on the composition and toxicity of cigarette mainstream smoke were evaluated by COGGINS *et al.* (247). Tobacco was fortified with three doses of triacetin (up to 10%) to produce sufficiently high levels of triacetin in the smoke. The cigarettes were prepared with two different triacetin levels in the cellulose acetate filters (target levels: 6% and 10%). The mainstream smoke of the test cigarettes was compared to cigarettes containing the level of triacetin in filters usually found in commercial products (inclusion level: 8%). The inclusion of triacetin in tobacco reduced almost all mainstream smoke constituents tested compared to control cigarettes. It also led to significant reductions of the mutagenicity response (Ames assay with S-9 activation) at the middle and high inclusion levels and produced a decrease of the cytotoxicity of cigarette smoke condensate. The different levels of triacetin in the filters showed no difference in mutagenicity and cytotoxicity of the particulate and gaseous phases of mainstream cigarette smoke. 90-day nose-only inhalation with rats showed no consistent differences in pulmonary physiology between the animal groups.

4.5.10. Ammonium compounds

A safety assessment of diammonium phosphate and urea, used in the manufacturing of cigarettes, was published by STAVANJA *et al.* in 2008 (484). In the course of the evaluation of the mainstream smoke toxicity of 95 additives (241), COGGINS *et al.* (250) investigated the effects of diammonium phosphate and ammonium hydroxide in experimental cigarettes. Their results showed that the addition of these compounds to cigarette tobacco, even at high inclusion levels, had - if any - minimal toxicological effects.
The alleged role of ammonium compounds in the availability of nicotine to smokers is discussed in Section 3.2. on page 419–421.

5. EPIDEMIOLOGICAL FINDINGS AND DATA OBTAINED BY THE BIOMONITORING OF SMOKERS CONSUMING CIGARETTES WITH AND WITHOUT ADDITIVES

Evidence for the firm relationship between cigarette smoking and various cancers and diseases of the respiratory and cardiovascular system has been demonstrated in numerous epidemiological studies, primarily in the United States, England and Canada (349). In the U.S., cased and flavored blended cigarettes are smoked almost exclusively. They contain dozens of additives. In England and Canada, mainly cigarettes are consumed made from "bright" Virginia tobaccos without tobacco additives. In 2000, one of the best selling brands in England contained only six additives, namely calcium carbonate, cellulose fiber, diammonium hydrogen phosphate, ethylene vinyl acetate copolymer, sorbitol and the di- and tri-potassium salts of citric acid (485). These additives were not added directly to the tobacco during cigarette manufacturing; much rather, they were used as binders and additives for the manufacturing of reconstituted tobacco sheet and cigarette paper.
The health risks associated with cigarette mainstream smoke are influenced by the kind of cigarettes, the number of cigarettes smoked during life time and the smoking topography of the consumer (puff volume, puff frequency, inhalation depth). The determination of biomarkers of exposure in smokers represents a possibility for evaluating the influence of these parameters on smoke uptake. Therefore, the comparison of biomarkers of exposure between consumers of additive free and additive containing cigarettes is an important approach to studying the effects of tobacco additives.
In several studies the effects of plain *vs.* filter cigarettes on lung cancer were investigated, in a few only the impact of cigarettes made from dark air cured tobaccos ("French" cigarettes) *vs.* "blond" cigarettes (U.S. blend cigarettes) (486–489). Epidemiological studies on the effects of additives on the health risks for smokers are very rare. One of the reasons is that only few additives, such as sugars, cocoa, licorice and humectants, are used in relatively large quantities in American blend cigarettes while the numerous aromatic additives are found in variable - generally minute - amounts and only in distinct brand specific formulas. Therefore, it is nearly impossible to evaluate the health effects of a specific single additive in a distinct market or country by epidemiological methods.
However, there is one additive suitable for investigating its health effects by epidemiology: menthol. Mentholated cigarettes are made from tobacco blends like those in U.S.

cigarettes. They contain humectants, sugars and other additives as well as menthol at a level of 0.3% to more than 1.5%. In some countries mentholated cigarettes have a market share sufficiently high to allow meaningful epidemiological studies. In the U.S., for instance, the market share of menthol cigarettes is around 26% (300).

5.1. American blend cigarettes vs. Virginia cigarettes

As mentioned earlier, contrary to cased and flavored U.S. blended cigarettes, "English" cigarettes (Virginia or "bright" cigarettes) are generally manufactured without any additives in the flue cured tobacco. In the United Kingdom, Canada and Australia the market share of these cigarettes is between 90% and 100%. On the other hand, there are many countries where virtually only cased and flavored blended cigarettes are smoked.

The outright comparison of the attractiveness of cased and flavored American blend cigarette brands to Virginia or Oriental brands (these types of cigarettes are free from added aroma and additives influencing the taste of smoke) is difficult. American blend, Virginia or Oriental brands are strongly preferred by smokers in distinctive markets. The "attractiveness" of a cigarette brand is an important factor in competing in the market place. In American blend cigarettes, it is achieved by the composition and quality of the tobacco blend together with the recipe of the applied casing and flavors. In Virginia and Oriental cigarettes, the composition and quality of the tobaccos is the most important factor. Brand name and package design also play a major role.

With the objective of investigating differences in the toxicity and carcinogenicity of cigarette mainstream smoke of both kinds of tobacco blends (cased and flavored U.S. blends *vs.* additive free Virginia blends) the evaluation of epidemiological data collected in U.S. blend and Virginia cigarette markets is expected to be useful for assessing the effects of additives on the health risks of smoking.

5.1.1. Biomonitoring studies in smokers consuming American blend cigarettes and Virginia cigarettes

Except for menthol (280, 333, 338, 490–493) we are not aware of any systematic investigation of the impact of tobacco additives on the levels of biomarkers of exposure, which could be used as an indicator of smoking intensity. Some indirect evidence, however, can be deduced from biomarker studies with additive free Virginia and cased and flavored American blend cigarettes.

Suitable data for comparison are available from a recent series of papers published by the Research and Development Group of Britisch American Tobacco (494–496). In two studies, "mouth level exposure" and biomarkers of exposure of selected smoke constituents were determined in similarly designed studies in Germany where primarily American blend cigarettes are smoked (495), and Canada where mainly Virginia cigarettes are consumed (496).

"Mouth level exposure" represents the amount of mainstream smoke per cigarette taken up into an individual smoker's mouth as a surrogate for what the smoker inhales. Smoke yield is determined by the smoker's individual smoking habit (puff volume, puff duration and puff number) and is, therefore, also called "human smoking yield". Mouth level exposure can be assessed by measuring the amount of particulate matter (or its components) retained on the cigarette filter. In addition, a calibration function, which relates the yield of a component in mainstream smoke to the amount retained on the filter, has to be determined for each brand in order to calculate mouth level exposure based on filter analysis. This method was described by ST. CHARLES *et al.* (497) and SHEPPERD *et al.* (498).

For the German study (495), smokers of cigarette brands with an ISO "tar" range per cigarette (73) of 1–2 mg (Group 1), 4–6 mg (Group 2) and 9–10 mg (Group 3) were recruited. Each group consisted of about 50 smokers. The groups investigated in Canada smoked cigarettes with "tar" yields of 4–6 mg (Group 1), 8–12 mg (Group 2) and 14–15 mg (Group 3). For comparing the results of smokers of American blend and Virginia cigarettes with comparable ISO "tar" and nicotine yields, Groups 2 and 3 of the German study and Groups 1 and 2 of the Canadian study were selected. Mouth level exposure and biomarker data of the groups are shown in Table 1.

The mouth level exposures for nicotine, estimated for a time interval of 24 hours, were only moderately different between the respective groups of smokers from Germany and Canada. This was also reflected by the biomarker data or urinary nicotine equivalents. Likewise, the biomarker levels for acrolein (3-hydroxypropylmercapturic acid) and pyrene (1-hydroxypyrene) showed no significant differences between the respective groups from Germany and Canada, confirming the results for nicotine (mouth level exposure and urinary equivalents). Only total NNAL excretion, the main metabolite of the tobacco specific *N*-nitrosamine NNK, was different between the smokers from Germany and Canada. This can be explained by the differing NNK yields in the smoke of German and Canadian cigarettes. The average NNK mouth level exposure in German smokers was 605 ng/24 hours (Group 2) and 1,177 ng/24 hours (Group 3). As it was not possible to establish the required calibration curve due to the very low levels of NNK in flue cured tobacco smoke the authors were not able to report data on NNK mouth level exposure for the smokers of Canadian style cigarettes. The levels for salivary cotinine and plasma cotinine showed no significant difference between German and Canadian smokers. The biomarker data in combination with the mouth level exposure data show that there was no substantial difference in smoking behavior between German and Canadian smokers though the type of cigarettes clearly differed (cased and flavored American blend cigarettes *vs.* additive free Virginia cigarettes). The data suggest that the use of tobacco additives in American blend cigarettes does not lead to a consistent increase in smoking intensity.

A similar conclusion was derived from a mouth level exposure study in eight countries (494). Differences in mouth exposure levels of "tar" and nicotine between American blend and Virginia cigarette smokers could mostly be explained by differences in the ISO smoke yields. The authors of the study had data available to compare the mouth level exposure of smokers of American blend and Virginia cigarettes within two of the eight

Table 1. Comparison of smoke doses determined by either mouth level exposure or biomarker measurements when smoking American blend or Virginia (flue cured) cigarettes [a].

	German study (495)		Canadian study (496)	
Blend type	American blend		Flue cured blend	
ISO "tar" band (mg/cig)	4 – 6 (Group 2)	9 – 10 (Group 3)	4 – 6 (Group 1)	8 – 12 (Group 2)
ISO yields				
ISO "tar" (mg/cig)	4.7	10.5	3.8	8.4
ISO nicotine (mg/cig)	0.45	0.83	0.44	0.90
ISO NNK (ng/cig)	11.5	28.8	< 8.0 (< LOQ)	< 8.0 (<LOQ)
ISO acrolein (µg/cig)	27.3	45.3	22.5	48.8
ISO pyrene (ng/cig)	33.0	49.4	25	41.1
Mouth level exposure data				
Nicotine (mg/24 h)	21.4 (19.1 – 23.7)	32.6 (29.4 – 35.8)	28.6 (25.5 – 31.8)	30.2 (27.3 – 33.1)
NNK (ng/24 h)	605 (538 – 673)	1177 (1057 – 1296)	not quantifiable	not quantifiable
Acrolein (µg/24 h)	1394 (1240 – 1549)	1920 (1720 – 2120)	1880 (1646 – 2114)	1773 (1600 – 1946)
Pyrene (ng/24 h)	1344 (1208 – 1481)	1914 (1727 – 2102)	1214 (1099 – 1330)	1263 (1148 – 1378)
Biomarker data				
Nicotine equivalents (mg/24 h) (biomarker for nicotine uptake)	13.4 (11.5 – 15.2)	18.1 (16.2 – 20.1)	14.5 (12.7 – 16.2)	15.1 (13.4 – 16.8)
NNAL (ng/24 h) (biomarker for NNK exposure)	295 (247 – 343)	489 (426 – 551)	213 (178 – 248)	176 (147 – 204)
3-Hydroxypropylmercapturic acid (µg/24 h) (biomarker for acrolein exposure)	1354 (1136 – 1572)	2028 (1761 – 2296)	1973 (1739 – 2207)	1868 (1614 – 2121)
1-Hydroxypyrene (ng/24 h) (biomarker for pyrene exposure)	262 (229 – 295)	331 (287 – 374)	334 (292 – 375)	276 (244 – 309)
Saliva cotinine (ng/ml) (biomarker for nicotine exposure)	240 (207 – 272)	356 (317 – 395)	266 (229 – 304)	298 (264 – 331)
Plasma cotinine (ng/ml) (biomarker for nicotine exposure)	192 (168 – 217)	280 (254 – 305)	242 (208 – 275)	254 (228 – 280)
Cigarettes per day	21.5 (19.1 – 23.2	22.4 (21.0 – 23.9)	18.8 (17.5 – 20.1)	17.9 (16.3 – 19.4)

[a] Numbers in brackets: 95 % confidence interval; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; Nicotine equivalents: sum of the amounts of nicotine, 3-hydroxycotinine, cotinine, nicotine-*N´*-oxide and the corresponding glucuronides

countries. In Australia, American blend cigarettes with 0.5 mg smoke nicotine/cig (according to ISO) gave a mean nicotine mouth level exposure per cigarette to ISO ratio of 2.27, and the corresponding Virginia cigarettes with 0.51 mg smoke nicotine/cig (according to ISO) showed a mean ratio of 2.44. In New Zealand, the mean ratio for American blend cigarettes (0.45 mg smoke nicotine/cig - ISO yield) was 2.62 and the corresponding value for Virginia cigarettes (0.47 mg smoke nicotine/cig - ISO yield) was 2.53. The differences between the ratios were statistically not significant in both instances. The authors stated that their mouth level exposure and biomarker data on blend differences were limited and so far not conclusive and recommended further work to be done in order to determine the impact, if any, of additives on cigarette smoke exposure.

5.1.2. Epidemiological evaluation of the influence of cigarette additives on health risks

In 2009, LEE *et al.* (238) tried to clarify in a multi-country approach the question whether there was an influence of additives on health risks by comparing the smoking related mortality rates (lung cancer and chronic obstructive pulmonary disease (COPD)) in countries that predominantly used tobacco additive free flue cured (Virginia) cigarettes with countries using cased and flavored blended cigarettes, such as U.S. cigarettes. Lung cancer and COPD were selected by the authors because they were major causes of smoking related death and their population attributable risk from smoking was very high. Ischemic heart disease also has a high incidence but a much lower population attributable risk from smoking because many

other factors are causally related.

LEE *et al.* selected the following countries for their investigation: Countries with a particularly high market share of cigarettes made from flue cured tobaccos: the United Kingdom, Canada and Australia; and countries where predominately U.S. style cased and flavored blended cigarettes were consumed: the United States, Germany, Denmark and Austria. These countries were chosen because it was possible to obtain the necessary data and the population did not include a large proportion of smokers of tobacco products other than cigarettes (499). Six 5-year periods were considered: 1971–1975, 1976–1980, 1981–1985, 1986–1990, 1991–1995 and 1996–2000. Data on mortality and on the prevalence of current smoking and ex-smoking were assessed for 5-year age groups from 35–39 up to 75–79. Data on cigarette consumption per smoker were not age specific, but were for the age range over 35. Mortality and population data were obtained from the World Health Organization Mortality Database of the WHO Statistical Information System (WHOSIS; www.who.int/whosis/en), now incorporated into the Global Health Observatory (GHO; www.who.int/gho/en). Relative risks for lung cancer in current smokers and ex-smokers were taken from the International Evidence on Smoking and Lung Cancer (IESLC) database (500, 501), which is based on data of all epidemiological lung cancer studies with over 100 cases published until the year 2000. All estimates selected by LEE *et al.* related to the period from 1971 to 2000 and were for all lung cancer types and all ages combined and were sex specific. Relative risks for COPD were derived from a literature search. Six studies (502–506) were found related to the countries and the time periods under investigation, providing 11 sex specific relative risk estimates for current smokers and 10 for ex-smokers.

With regard to lung cancer mortality rates, LEE *et al.* reported for all age groups and periods combined that the ratio between cased-flavored blended U.S. style cigarettes and flue cured Virginia cigarettes was 0.97 (95% CI = 0.76–1.23) for males. In females, mean lung cancer mortality rates tended to rise over the period studied in all groups for both U.S. blended cigarette and Virginia countries. The risk of dying from lung cancer was somewhat lower for smokers in the U.S. blend cigarette countries, particularly at ages 50 to 64 and 65 to 79 years. However, these differences were not statistically significant. Overall, the risk ratio for U.S. blended *vs.* Virginia cigarettes for females was 0.80 (95% CI = 0.31–2.09).

The mean COPD mortality rates for U.S. blend smokers were initially (for the time period 1971–1975) lower than for Virginia smokers, with the difference diminishing over time so that by the period 1996–2000 the rates in the U.S. blend and flue cured cigarette countries were similar. Overall, the U.S. blend/Virginia ratio for COPD in males was estimated to be 0.80 (95% CI = 0.49–1.31).

Smoking cessation increased over the time of observation and was somewhat lower in countries, where U.S. blend cigarettes were smoked. However, the difference to flue cured cigarette countries was not statistically significant.

LEE *et al.* pointed out that for all countries included in the study fairly complete estimates by sex, 5-year period and 5-year age group could be calculated for the prevalence of current and ex-smokers and for lung cancer and COPD mortality rates. Data were also available for daily cigarette consumption per smoker for ages over 35. However, according to LEE *et al.*, any comparison between countries suffered from several inherent weaknesses. First, it was ecological (based on populations) rather than epidemiological (based on individuals). Second, it did not take into account potential confounding variables. Third, each comparison was based on a statistical test using only seven data points, one per country. Fourth, the data may have suffered from various sources of inconsistency by time period and country. Fifth, the estimates when compared may all have been subject to sampling variation. LEE *et al.* summarized that their study not only suggested that there were no major differences in lung cancer and COPD risks between U.S. blend and Virginia cigarettes but also highlighted some differences in lung cancer and COPD rates between countries that remained unexplained by simple adjustment for prevalence of current and former smoking and consumption per smoker. The fact that no major differences in the risks of these two important smoking-related diseases were observed between the two types of cigarettes suggests that additives added to U.S. blend cigarettes do not have a major effect on these health risks associated with cigarette smoking.

The conclusion drawn by LEE *et al.* that the tobacco additives used in cigarette manufacturing had little or no effect on the health risks of smoking may be challenged for another reason. It can be hypothesized that different kinds of cigarettes are consumed in different ways seemingly demonstrating the absence of health risks from additives. However, the studies on biomarkers of exposure, presented in Section 5.1.1. (on page 460–461) strengthen the body of evidence that cigarette additives have no major effect on smoking topography.

5.2. "French" (dark) cigarettes vs. American blend ("blond") cigarettes

For the comparison of these two types of cigarettes only epidemiological data are available but no results from biomonitoring studies or the assessment of smoking topography.

In 1985, BENHAMOU *et al.* (486) investigated the lung cancer risk of smokers of "French" (dark) and American blend (called "blond" or "light" in France) cigarettes and consumers of hand-rolled tobacco. Contrary to American blend cigarettes "French" cigarettes are made from dark air cured tobaccos without additives except humectants. A case-control study with 1,625 histologically confirmed lung cancer cases and 3,091 controls was conducted in France from 1976 and 1980. In male smokers, a significant difference in the risk for cancers of the KREYBERG I category (507) was found between consumers of dark cigarettes (RR = 18.1 compared to non-smokers) and American blend cigarettes (RR = 4.9 compared to non-smokers). According to the authors, the result fits the hypothesis of more harmful effects of dark tobacco compared to light tobacco (508), which was supported by an epidemiological case control study conducted in Cuba (509). It showed that, compared to non-smokers, the relative risk for lung cancer was higher for consumers of

cigarettes made from dark air cured tobaccos than from American blends (RR = 14.3 *vs.* 11.3 for men and 8.6 *vs.* 4.6 for women).

Two years later BENHAMOU *et al.* (487) assessed the lung cancer risk of women using data of their French case-control study. 96 women with histologically confirmed lung cancer and 192 matched controls were included in the study. A significantly increased lung cancer risk was found for women smoking also dark "French" besides "blond" cigarettes, and an even higher risk for those smoking dark cigarettes exclusively, compared to smokers of "blond" cigarettes only. Significance was lost when either daily consumption or duration of smoking or age at first cigarette were taken into account.

In a third paper, published in 1989, BENHAMOU *et al.* (488) used data from their case-control study to evaluate how changes in smoking habits, e.g., switching from dark to American blend cigarettes or stopping to smoke, had an effect on lung cancer risk, especially after smoking cessation. After not smoking for 20 years, the lung cancer risk remained 2-fold in people who had only smoked cigarettes made from dark "French" tobaccos compared to smokers of American blend cigarettes. In the analysis, a non-significant lower lung cancer risk was observed in former smokers, who had switched during their smoking career from dark "French" to American blend cigarettes.

In 1994, the effects of tobacco type (American blend *vs.* "French" dark air cured) on lung cancer risk in males were evaluated by BENHAMOU *et al.* (489) in an additional paper. For this purpose, the data of the case-control study conducted between 1976 and 1980 were used together with information on the "tar" content of the most popular "French" cigarette brands made from dark tobaccos. "Tar" values of the imported U.S. blend cigarettes were not considered. After adjustment for age, cigarettes smoked per day and years of smoking an increase of lung cancer risk of borderline significance was found in men when the "French" cigarettes were compared to imported cased and flavored U.S. blend cigarettes (RR = 2.6, 95% CI = 0.9–7.7).

According to the findings discussed above, tobaccos, and not the additives in tobacco, contribute predominantly to the smoking related health risk, lung cancer. The smoke of "French" dark cigarettes differs from U.S blend cigarettes in taste, aroma and character. For instance, the mainstream smoke pH of dark cigarettes is slightly alkaline while the pH of U.S. blend cigarette smoke is slightly acidic.

The difference in observed lung cancer risk may also be explained by different smoking habits. There are no data of biomonitoring studies, such as nicotine and nicotine metabolite levels excreted in urine or carbon monoxide levels in expired air, which reveal how smoking habits are influenced by the type of tobacco smoked. The depth of inhalation, as recorded in a rather poor way in the last study (489), is not a suitable indicator for a specific smoking habit. In our opinion, the personal judgment of participants regarding inhalation as "none", "moderate" or "deep" without any validation has little value. Participants' feelings are non-comparable and quantitatively unreliable. However, the studies of BENHAMOU *et al.* (486–489) may be seen as a strong indicator that the type of tobacco itself plays the main role for the health risks of smoking and not the additives used in manufacturing. This assumption is supported by the comparison of the carcinogenic effects of the mainstream "tar" of cigarettes, made from commercial Columbian black air cured tobaccos, and U.S. blend cigarettes in a mouse skin painting assay (508), and the epidemiologic study on smoking and lung cancer from Cuba (509).

5.3. Menthol cigarettes

The effect of menthol as tobacco additive on the risk of suffering from smoking related diseases can be evaluated in epidemiological studies comparing consumers of mentholated and non-mentholated cigarettes. For the precise assessment of the influence of menthol smoking behavior and smoke uptake by consumers must be understood. In this section, published studies on the smoking behavior (smoking topography) of menthol smokers and the influence of menthol on the uptake and metabolism of smoke components, and epidemiological studies comparing smoking related health risks in users of mentholated and non-mentholated cigarettes are reviewed.

5.3.1. Influence of mentholated cigarettes on smoking topography and biomarkers of exposure

In his review, HECK (267) presented an overview of the published studies concerning the influence of cigarette mentholation on smoking topography (smoking habits) and the levels of biomarkers of exposure in smokers. The determination of biomarker levels is important for the evaluation of the impact of menthol on smoke uptake. If uptake by menthol and non-menthol smokers was comparable an influence of menthol on smoking habits would not need to be taken into account (510).

It was speculated by WAGENKNECHT *et al.* (511), GARTEN and FALKNER (512), and AHIJEVYCH and GARRETT (513) that smoking mentholated cigarettes might result in increased nicotine intake due to the desensitizing "anesthetic" effect of menthol allowing deeper inhalation. However, based on the results of the study conducted by GREEN and MCAULIFFE (514) it can be assumed that menthol uptake by smoking mentholated cigarettes is too low for this effect. NIL and BÄTTIG (515) used the tidal carbon monoxide boost after smoking and showed that mentholated cigarettes were smoked less intensely than cigarettes of other taste categories (cigarettes made from dark air-cured tobaccos and American blend cigarettes).

The relationship between self-reported cigarette smoking rates and salivary cotinine concentration and the degree of nicotine dependence was studied by AHIJEVYCH and WEWERS (516) in black women. The mean salivary cotinine concentration in menthol smokers (394 ng/mL) was not significantly different from non-menthol smokers (369 ng/mL). Menthol cigarette smokers tended to have a non-significantly higher smoking rate compared to non-menthol users. Underreporting of cigarette consumption, defined as a cotinine level > 25 ng/mL/cig, ranged from 86% among light smokers to 70% among moderate smokers and 21% among heavy smokers. The authors concluded that there was a need to continue exploring smoking topography, nicotine metabolism, and the effects of menthol. It must be pointed out that the cohort of menthol

cigarette smokers consisted of 130 subjects while the non-menthol cigarette smokers numbered only 12. The study is as good as invalidated by the alarming rates of underreporting and the imbalance in participant apportionment.

In a racially mixed group of women, who were regular smokers of either menthol or non-menthol cigarettes, AHIJEVYCH *et al.* (517) compared in 1996 smoke constituent exposures by measuring CO boosts in expired air and nicotine boosts in venous blood (combining nicotine and cotinine levels). Both in black and white women, lower end-expired carbon monoxide boosts were observed for menthol cigarette smokers (combined mean = 6.5 ppm) than for non-menthol cigarette smokers (10.6 ppm). Differences in mainstream carbon monoxide values obtained by machine smoking according to ISO (73) and FTC (142) as well as differences in smoking behavior could not explain this finding. The authors concluded from the results of this study that the smoke of mentholated cigarettes may be inhaled less deeply than the smoke of menthol free cigarettes. Like the earlier study of AHIJEVYCH and WEWERS (516) this study as well suffers from an inadequate sample: 18 blacks (8 of them menthol smokers) and 19 whites (10 of them menthol smokers).

In a third study, published in 1999, AHIJEVYCH and PARSLEY (518) investigated differences in smoke constituent exposure (plasma nicotine and cotinine, exhaled CO) between female menthol and non-menthol smokers in a clinical study. 48 Afro-american women (27 of them menthol smokers) and 47 white women (22 of them menthol smokers) participated in the study. Afro-american women smoked significantly fewer cigarettes per day but had higher blood cotinine levels compared to white women. Menthol smokers had significantly larger puff volumes and higher cotinine levels. The results of this third study were in contrast to previous studies (515, 517), which had shown that smokers of mentholated cigarettes inhaled the smoke to a lesser degree and that these cigarettes were smoked less intensively. A possible explanation for the new results of AHIJEVYCH and PARSLEY (518) may be the influence of study set-up. Contrary to other studies, the participants in this clinical study smoked their cigarettes in a laboratory under controlled conditions. It is well known that experimental conditions may influence individual smoking habits and, consequently, the results of a study (519).

This study of AHIJEVYCH and PARSLEY (518) was heavily criticized by HECK (267). The participants of the study were not instructed to refrain from smoking before the session; the self reported number and kind of cigarettes smoked before as well as during the sessions were not confirmed. In the light of these shortcomings of the study set-up HECK stated that "*the authors' attribution of the differences seen to menthol is difficult to accept*". Due to the half-life time of cotinine in the body of about 17 hours the higher plasma cotinine values found in mentholated cigarette smokers may include a substantial contribution from uncontrolled tobacco consumption prior to the laboratory smoking sessions.

MILLER *et al.* (520) evaluated the effect of menthol in cigarettes on inhaled puff volume and carbon monoxide exhalation in 12 overnight smoking abstinent male Afro-american subjects, of whom 6 were regular menthol smokers. After each subject had smoked two consecutive experimental cigarettes containing 0, 4 or 8 mg menthol, in three separate controlled-dose smoking sessions spaced one week apart, using a mechanical device that allowed smokers to take puffs at 30-sec intervals until they had inhaled a volume of 600 mL per cigarette, breath samples were collected for carbon monoxide determination. Menthol in the cigarettes had no significant effect on puff volume, puff number, blood pressure or heart rate. However, the authors reported that at the highest menthol level, the carbon monoxide level in exhaled breath was significantly higher (8.1 ppm) than after smoking cigarettes containing 4 mg menthol (6.1 ppm) or cigarettes with no menthol (5.6 ppm). MILLER *et al.* concluded from their results that the addition of menthol to cigarettes influenced the absorption of one constituent of cigarette smoke, carbon monoxide, and speculated that the absorption of other smoke constituents may be increased in a similar way.

According to HECK (267) the finding of this small study (two groups of 6 participants each), that menthol increased carbon monoxide transfer across the respiratory membranes, is not supported by biological plausibility. In addition, the chosen experimental set-up was far from reality and interference could not be excluded (519). In our opinion, the way the test cigarettes were prepared could also have influenced the results: An alcoholic menthol solution was injected into the tobacco rod of a commercial cigarette brand. This manipulation may have produced unbalanced smoke taste and, consequently, influenced the smoking habits of the test persons.

The potential of menthol to affect the depth of inhalation and the retention of inhaled cigarette smoke was investigated by JARVIK *et al.* (521). Ten male menthol cigarette smokers and ten male smokers of menthol free cigarettes participated in the study. Half of the subjects were whites and half blacks. The two styles of commercially available cigarettes (menthol and non-menthol versions of a brand family) were equivalent with regard to smoke nicotine, carbon monoxide and "tar" yields. Smoking was done in two sessions in a laboratory under controlled conditions and smoking parameters were recorded with a pressure transducer. The equipment corresponded to that used in the study of MILLER *et al.* (520). Mentholated cigarettes were found to produce significantly smaller mean puff volumes and significantly smaller mean puff numbers, while other smoking parameters such as puff duration, intervals between puffs and lung retention times were similar for both types of cigarettes. No indication of increased intensity of puffing was found with cigarette mentholation. Although no significant difference in the end-expired carbon monoxide boost was evident between smokers of menthol free and mentholated cigarettes, both the end-expired carbon monoxide boost and blood carbon monoxide hemoglobin levels were significantly higher in smokers of menthol cigarettes than after smoking menthol free cigarettes when expressed relative to the cumulative puff volumes inhaled. Based on this finding JARVIK *et al.* speculated that smoking mentholated cigarettes increased either the diffusivity of the alveolar capillary membranes for carbon monoxide or the affinity of hemoglobin for carbon monoxide. This speculation, however, seems highly

improbable and without biological plausibility. The critique of the experimental set-up and its influence on the smoking behavior of the subjects is explained above.

McCarthy *et al.* (522) of the same research group as Miller *et al.* (520) and Jarvik *et al.* (521) reported in a laboratory study with 29 males (18 of them preferring non-mentholated and 11 menthol cigarettes) that smokers of mentholated cigarettes took fewer puffs with smaller puff volumes compared to smokers of non-mentholated cigarettes, especially when the cigarettes were smoked rapidly. For evaluating the smoking topography of the subjects an experimental set-up was used like the one in the other studies of this research group (520, 521). The authors found no significant menthol associated effects on heart rate, blood pressure and exhaled carbon monoxide levels under the conditions of the study. They concluded that carbon monoxide exhalation and heart rate were not reduced proportionally by the less intense puffing behavior observed in menthol smokers. This was taken as an indication that menthol in cigarette smoke increased the efficiency of carbon monoxide uptake in the respiratory tract. This conclusion of McCarthy *et al.* (522) was criticized by Heck (267) as "*purely speculative*" owing to several shortcomings in the study design. The cigarettes used were two commercially available brands, one mentholated and the other non-mentholated, manufactured by two different cigarette companies. The mentholated brand delivered 13% more carbon monoxide than the non-mentholated brand, using the FTC smoking regimen (142). There was no pre-study smoking abstinence period nor was there any validation of the sensitivity of the cardiovascular functional assessment protocols in registering the relatively minor reported differences in the intake of the purported active smoke constituents.

In 1996, Clark *et al.* (523) evaluated the effect of menthol on the biomarkers of tobacco smoke exposure among black and white smokers. 161 male and female subjects participated in the laboratory study. Serum cotinine levels were reported to be significantly higher in menthol smokers (478.2 ng/mL) than in smokers of non-mentholated cigarettes (349.1 ng/mL). The mean unadjusted expired carbon monoxide level of menthol smokers was not significantly different from the level of non-menthol smokers (40.3 *vs.* 35.8 ppm). However, menthol in cigarette smoke had been described as a significant contributor to expired carbon monoxide levels after adjustment for the number of cigarettes smoked per day and the length of each cigarette smoked (521, 522). Clark *et al.* (523) concluded that menthol in cigarette smoke was associated with higher serum cotinine levels and higher uptake of carbon monoxide. Consequently, the use of mentholated cigarettes might be associated with increased health risks of smoking. According to Heck (267), a number of shortcomings in the design of the study call into question the experimental support for the conclusions of Clark *et al.* For instance, serum cotinine levels and expired breath carbon monoxide after smoking may actually have been influenced by the fact that participants were allowed to engage in uncontrolled smoking 1 hour prior to the laboratory session. Elimination half period for carbon monoxide from the body is between 4 and 6 hours (524) and for cotinine in plasma about 17 hours (525).

Investigating olfactory thresholds for nicotine and menthol Rosenblatt *et al.* (526) measured exhaled carbon monoxide in five male smokers each of mentholated and non-menthol cigarettes. Carbon monoxide in menthol cigarette smokers (16.2 ppm) was significantly lower than in non-menthol cigarette smokers (24.4 ppm).

In a small study, Pritchard *et al.* (527) detected no significant differences in tidal breath carbon monoxide boosts between 10 smokers of mentholated and non-mentholated nicotine free cigarettes.

In 2010, Wang *et al.* (528) compared the exposure of smokers of mentholated and non-mentholated cigarettes to nicotine and carbon monoxide. They found that smoking mentholated cigarettes did not increase daily exposure to smoke constituents when measured as serum cotinine, nicotine equivalents in 24-hour urine (sum of cotinine, cotinine-*N*-glucuronide, *trans*-3'-hydroxycotinine and its glucuronide and nicotine-*N*-glucuronide) and carboxyhemoglobin. This confirmed the results obtained by Muscat *et al.* (333) that mentholization of cigarettes did not affect the uptake of nicotine by smoking.

In 2011, Nelson *et al.* (529) measured the "tar" and nicotine mouth level exposure in smokers of U.S. commercial mentholated and non-mentholated cigarettes using the method described by St. Charles *et al.* (497) and Shepperd *et al.* (498). They reported trends towards slightly lower "tar" and nicotine mouth level exposures with mentholated cigarettes than non-mentholated cigarettes of comparable "tar" yield. In the authors' view the study demonstrated that cigarette mentholation was not associated with increased "tar" and nicotine mouth level exposure in smokers.

Ashley *et al.* (530) examined the effect of low and high menthol levels in experimental cigarettes on "tar" and nicotine mouth level exposures in both regular and occasional smokers of mentholated low "tar" cigarettes in Japan and Poland. These countries were selected because the market share of mentholated cigarettes in 2006 was relatively large (19.4% in Japan, and 12.8% in Poland). "Tar" yields were 1 mg and 4 mg according to ISO (73). Menthol inclusion reflected the lowest and highest levels found in 2006 for this kind of cigarettes in the two countries (8.2–13 mg/cig in Japan, and 4.8-6.3 mg/cig in Poland). No marked menthol induced increases in "tar" and nicotine mouth level exposures were seen independent of menthol load. Consequently, the data of Ashley *et al.* do not support the assumption that mouth level exposure to "tar" and nicotine is increased in smokers of menthol cigarettes (301, 303, 513).

Pickworth *et al.* (531) found no statistically relevant differences in exhaled carbon monoxide boosts after smoking mentholated and non-mentholated cigarettes of different "tar"/nicotine categories. No menthol related independent effects on heart rate or systolic and diastolic blood pressure were identified.

According to Patterson *et al.* (532) smokers of mentholated cigarettes exhibited higher baseline plasma cotinine levels while there was no significant difference in nicotine uptake per cigarette smoked relative to smokers of non-mentholated cigarettes.

In a 3-week crossover study with 14 smokers (one-half of them white and one-half Afro-american), Benowitz *et al.*

(533) compared the effect of smoking non-menthol or mentholated cigarettes under comparable conditions on the systemic absorption of tobacco smoke toxins and on the metabolism of nicotine. Subjects were randomly assigned to smoking mentholated or non-mentholated cigarettes for one week, followed by crossover to the other type of cigarettes for one week. The uptake of nicotine and carbon monoxide was not affected by the mentholation of cigarettes but a significant retardation of nicotine metabolism was observed in menthol smokers, which was assumed to be the consequence of slower oxidative conversion to cotinine and slower formation of glucuronide conjugates. As a result, menthol-impaired nicotine metabolism may have enhanced systemic nicotine exposure. BENOWITZ *et al.* also measured menthol excretion by smokers and estimated with the help of data obtained after oral menthol administration that on average 20% of menthol contained in each cigarette (about 0.6 of 3 mg) were absorbed systemically by the smoker. BENOWITZ *et al.* concluded that their findings "*do not support the hypothesis that mentholated cigarette smoking results in a greater absorption of tobacco smoke toxins*" and show that "*...mentholated cigarette smoking enhances systemic nicotine exposure.*"

HECK (267) characterized the findings of BENOWITZ *et al.* (533) as "*consistent with the weight of epidemiological evidence ..., which indicates that menthol cigarettes are no more harmful than are non-mentholated cigarettes*".

In an additional paper, BENOWITZ *et al.* (534) suggested urinary menthol as a biomarker of the use of mentholated cigarettes. Concentrations of menthol glucuronide, the main metabolite of menthol excreted in urine, nicotine together with its metabolites (expressed as nicotine equivalents), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and polycyclic aromatic hydrocarbon (PAH) metabolites were measured in the urine of 60 menthol and 67 non-menthol cigarette smokers (white or Afro-american). Urinary menthol was measurable in (only) 82% of menthol smokers (geometric mean: 4.8 µg/mg creatinine; range 2.3–12.9) but also in 54% of non-menthol smokers (geometric mean: 2.1 µg/mg creatinine; range 0.5–7). Among menthol smokers, urinary menthol was highly correlated with nicotine equivalents, NNAL and PAHs. In a multiple regression model nicotine equivalents, but not menthol, were significantly associated with NNAL and PAHs.

Already the measured urinary menthol levels render this study highly questionable. While 18% of menthol smokers with no urinary menthol may be explained by feeble inhalation behavior, the detection of urinary menthol in 54% of non-menthol smokers (with considerable overlapping of the frequency histograms of menthol and non-menthol smokers) points at massive distortions of participants' starting status. Menthol is present in numerous consumer products, such as dental hygiene articles, foods, beverages, candies and chewing gums, and pharmaceutics. The authors were aware of this and accounted for all potential sources by forming a single menthol exposure score. In addition, the short half-life of menthol in the human body (75 min) weakens the value of urinary menthol glucuronide as a biomarker for mentholated cigarette use. We believe that urinary menthol is a uncertain biomarker, especially with persons consuming mentholated tobacco products at a low rate.

ST. CHARLES *et al.* (497, 535, 536) found no relevant differences in smoking topography as measured by increased inhalation volume or lung exposure time between smokers of mentholated and non-mentholated cigarettes. This was valid for cigarette brands of different FTC "tar" categories, 1–3 mg, 4–6 mg, 7–12 mg and 13+ mg per cigarette, smoked in the study.

RICHIE *et al.* (490) compared total urinary 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) - the main metabolite of the lung carcinogen, 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) - in black and white smokers to examine the hypothesis that racial differences in lung cancer risk might be related to differences in metabolism. The urinary ratio between free NNAL and its glucuronide was significantly higher in black than in white smokers. The authors suggested that their results indicated that black smokers' metabolism was less efficient in detoxifying NNK as none of the observed differences could be explained by dissimilarities in exposure, or other sociodemographic or dietary factors. No evidence was found that the racial difference was due to the preference of blacks for smoking mentholated cigarettes.

In 2009, MUSCAT *et al.* (333) published a community-based, cross-sectional study with 525 black and white smokers to examine the levels of biomarkers of exposure in smokers of mentholated and non-mentholated cigarette brands. Urinary and plasma cotinine, plasma thiocyanate and total urinary NNAL (sum of free NNAL and NNAL glucuronide) were determined. In regression models that adjusted for daily cigarette use no significant differences between black and white menthol smokers were observed in the levels of any of these biomarkers. However, while mean total NNAL amounts were similar for smokers of both kinds of cigarettes, the ratio of NNAL glucuronide to free NNAL in urine was significantly lower in smokers of mentholated compared to non-mentholated cigarettes both in whites (minus 34%) and blacks (minus 22%) and both in males and females. MUSCAT *et al.* hypothesized that menthol inhibits, or competes with, the glucuronidation of NNAL in the human liver and, thereby, impairs the urinary clearance of NNAL from the body.

In 2011, CARABALLO *et al.* (537) compared the serum cotinine concentrations of white and black smokers of mentholated and non-mentholated cigarettes. Serum cotinine concentrations were measured in 1,943 smokers participating in the 2001–2006 U.S. National Health and Nutrition Examination Surveys (NHANES) (538). Smoking a menthol rather than a non-menthol cigarette brand was not associated with higher mean serum cotinine concentrations in either black or white smokers. The higher levels of cotinine observed in black compared to white smokers could not be explained by their preference for menthol cigarette brands.

Recently, HECK (492) investigated the degree of smoke exposure from mentholated and non-mentholated cigarettes. 112 male and female subjects participated in the study smoking commercial cigarettes of similar "tar" yields of 9–10 mg according to the FTC smoking regimen (142). Study subjects smoked their preferred menthol or non-menthol cigarette brands at home in their normal way. Both blood sampling and 24-hour urine collection were performed twice spaced one week apart. Blood carboxyhemo-

globin (COHb), urinary nicotine metabolites and urinary total NNAL were measured and showed no statistically significant differences between mentholated and non-mentholated cigarette smokers. The biomarker levels for nicotine and NNK were statistically lower in white smokers of mentholated compared to non-mentholated cigarettes. HECK concluded that the smoking of menthol and non-menthol cigarettes of similar machine generated smoke yields resulted in essentially identical levels of biomarkers of cigarette smoke exposure.

In his review, HECK (267) concluded from the studies reviewed in 2010 with regard to menthol and biomarkers: "*The body of available studies on menthol and smoking topography do not provide convincing support for the hypothesis that menthol cigarette smoke is inhaled more intensely than is the smoke of regular cigarettes. The findings of studies to date are mixed, and the outcomes of diverse experimental attempts to measure human smoking topography may be method-dependent. ... Some studies reported to date have reported differences between menthol and non-menthol smokers' biomarkers levels, most consistently as higher levels of nicotine or the nicotine metabolite cotinine among menthol smoking subjects. However, the weight of available evidence to date does not support an expectation that elevations in exposure to smoke constituents that are believed to be significant in the etiology of smoking-related diseases results from the use of menthol as a cigarette flavoring ingredient* [(333, 490, 492, 533)]. *This conclusion is consistent with the body of complimentary epidemiological and smoking topography literature reviewed above. Further research into the potential of menthol to affect the metabolic disposition of biologically-significant smoke constituents is indicated.*"

Similar conclusions concerning biomarkers of exposure among smokers of mentholated cigarettes were drawn in the Menthol Report of ALTRIA CLIENT SERVICES (280) and in the U.S. Industry Menthol Report (338).

The TPSAC (339) concluded in their report submitted to the FDA in 2011: "*The evidence is insufficient to conclude that it is more likely than not that menthol smokers inhale more smoke per cigarette or they are exposed to higher levels of nicotine and other tobacco toxins.*"

5.3.2. Epidemiological studies of the use of mentholated vs. American blend cigarettes

The review by HECK (267) of menthol as an ingredient in cigarettes includes published epidemiological studies of health risks related to the consumption of mentholated cigarettes. In the following, these are summarized together with HECK'S conclusions.

Altogether, 13 epidemiological studies were reviewed and evaluated. Eight of them were case-control studies (539–546), two cohort studies (288, 547), two prospective studies (328, 548) and one a nested case-control study (549). Except one study, which was done in Germany (545), all studies came from the U.S. The risks for various smoking related diseases (cancers, such as lung cancer, esophageal cancer and oropharyngeal cancer, and cardiovascular diseases) were assessed.

The influence of cigarette mentholation on lung cancer risk was investigated by KABAT and HEBERT (540), SIDNEY *et al.* (288), FRIEDMAN *et al.* (547), BROOKS *et al.* (543), STELLMAN *et al.* (544), JÖCKEL *et al.* (545), MURRAY *et al.* (328) and ETZEL *et al.* (546). With the exception of the study of SIDNEY *et al.* (288) no study showed an increase of lung cancer risk among smokers of mentholated cigarettes compared to non-mentholated cigarettes, neither for men nor for women. In their study, STELLMAN *et al.* (544) reported that lung cancer risks were similar for white and black U.S. smokers with comparable smoking habits with the exception of a possibly increased risk for blacks, who were very heavy smokers of mentholated cigarettes.

SIDNEY *et al.* (288) reported a statistically significant increase of the relative risk for lung cancer in menthol cigarette smoking men to 1.45 (95% CI = 1.03–2.02), after adjustment for age, race, education, smoking duration, etc., compared to male smokers of non-mentholated cigarettes. For female menthol smokers the lung cancer risk of 0.75 (95% CI = 0.51–1.11) did not differ significantly from that of matched female smokers of non-mentholated cigarettes. FRIEDMAN *et al.* (547) could not confirm the results of SIDNEY *et al.* (288). He made the comment that the association of mentholation with lung cancer among male smokers found by SIDNEY *et al.* may have been merely a chance finding, particularly as it was absent in women and had not been reported elsewhere.

In 2008, ETZEL *et al.* (546) reported a case-control study comparing non-menthol with menthol and former menthol cigarette smokers, which showed no significant excess risk of lung cancer among former or current menthol cigarette smokers. Using study data, the authors developed and validated a risk prediction model for lung cancer that was specific for Afro-americans and, therefore, more precise in predicting risks in this population.

In addition to the epidemiological studies on mentholated cigarettes and lung cancer, which were reviewed by HECK (267), BLOT *et al.* (337) published in 2011 the results of a large prospective study with 85,806 racially diverse adult smokers classified by preference for menthol *vs.* non-menthol cigarettes. In a nested case-control analysis of 440 lung cancer patients and 2,213 matched controls, the authors estimated odds and hazard ratios of lung cancer incidence and mortality related to mentholated cigarette preference.

After grouping smokers by daily consumption (<10, 10–19, and ≥ 20 cigarettes), lung cancer incidence, compared to never smokers, was lower in each group of menthol smokers than in the respective group of non-menthol smokers; for <10 cigarettes: OR = 5.0 *vs.* 10.3, for 10–19 cigarettes: OR = 8.7 *vs.* 12.9, and for ≥ 20 cigarettes: OR = 12.2 *vs.* 21.1. The pattern was mirrored for lung cancer mortality; for <10 cigarettes: OR = 4.6 *vs.* 9.9, for 10–19 cigarettes: OR = 8.3 *vs.* 14.2, and for ≥ 20 cigarettes: OR = 13.9 *vs.* 16.1.

In a multivariable analysis adjusted for pack-years of smoking, menthol cigarette smokers showed lower lung cancer incidence and mortality than smokers of non-menthol cigarettes: hazard ratio of incidence = 0.65, 95% CI = 0.47–0.90, and hazard ratio of mortality = 0.69, 95% CI = 0.49–0.95). The authors concluded that "*the findings suggest that menthol cigarettes are no more, and perhaps less, harmful than non-menthol cigarettes*."

In 2011, LEE (550) reviewed systematically the epidemiological evidence for lung cancer risk in smokers of mentholated and non-mentholated cigarettes. The studies selected for the review had to satisfy several conditions: They had to be based on research on humans, had to be of cohort or case-control design, had to consider any form of lung cancer outcome, and had to provide risk estimates comparing mentholated and non-mentholated cigarette smokers. Eight studies were identified meeting the selection criteria (288, 328, 540, 542–546). In summary, LEE concluded: "*While there are some weaknesses in the studies presenting data .... the evidence taken as a whole is certainly consistent with the addition of menthol to tobacco having no effect on the lung carcinogenicity of cigarettes. The much greater preference for mentholated cigarettes in Black people in the United States cannot possibly explain their higher lung cancer risk, which in any case is evident only in men.*"

ROSTRON (551) conducted a survival analysis of participants in the 1987 U.S. National Health Interview Survey (NHIS) Cancer Control Supplement, who were followed for mortality through the linkage with the U.S. National Death Index (552–555). The NHIS is a nationally representative household health survey of the U.S. civilian non-institutionalized population and is conducted by the National Center for Health Statistics (552). 202,043 NHIS participants aged over 18 completed the 1987 Cancer Control Supplement, which asked participants about their cancer risk factors, including smoking, and recorded information about menthol cigarette smoking. 6,073 of the participants were smokers at baseline; 1,417 of them were smokers of mentholated cigarettes. 3,690 were identified as non-menthol smokers, and 966 had unknown cigarette preference. The results of the evaluation were presented in detail by ROSTRON in tables. A lower risk of lung cancer mortality was found for menthol smokers at ages of 50 and over compared to non-menthol smokers, based on over 20 years of follow up. The mortality hazard ratio of mentholated to non-mentholated cigarette consumption was 0.59 (95% CI = 0.37–0.95). A similar situation was not observed with other causes of tobacco related mortality. The mortality hazard ratio of mentholated to non-mentholated cigarette consumption for all causers of mortality, net of lung cancer, was found to be 0.97 (95% CI = 0.84–1.12). ROSTRON pointed out that a major limitation of his study was the limited number of lung cancer deaths in the sample of age 50+ (menthol smokers: 29; non-menthol smokers: 174). Other limitations were due to the information available in the 1987 NHIS Cancer Control Supplement. For instance, there was no information about the number of years of regular menthol cigarette smoking prior to 1987. According to ROSTRON, the reasons for the observed association of mentholated cigarette smoking and decreased risk of dying from lung cancer compared to other kinds of cigarettes were not clear.

In our opinion, there is no plausible explanation for the result concerning lung cancer reported by ROSTRON (551). The results are probably not an effect of the additive menthol in cigarettes. However, the study confirmed the findings of the study by BLOT *et al.* (337) and the review by LEE (550) concluding that there was no substantial difference in lung cancer risk for consumers of mentholated and non-mentholated cigarettes. The higher lung cancer rate in blacks could not be explained by their greater preference for mentholated cigarettes. This statement by LEE (550) was in line with the lung cancer risk prediction model developed by ETZEL *et al.* (546).

In a hospital based case-control study of esophageal cancer in menthol compared to non-menthol smokers, HEBERT and KABAT (539) found inconsistent results, namely a decrease in risk of marginal significance in male short-term (< 10 years) menthol smokers *vs.* never menthol smokers (OR = 0.50; 95% CI = 0.23–1.07) and no change in risk for menthol smokers of longer duration. For females, logistic analysis showed a marginally significant increase of risk for longer menthol use (OR = 2.30; 95% CI = 0.93–5.72).

In 1994, KABAT and HEBERT (541) investigated in another hospital based case-control study the association of oropharyngeal cancer and smoking of mentholated cigarettes. No difference in overall risk was found between the use of mentholated and non-mentholated cigarettes either for males or for females. In an analysis by subsite, smoking mentholated cigarettes was positively associated only with cancer of the pharynx in males, although the magnitude of the association was small. According to the authors these results indicated that the use of mentholated cigarettes was unlikely to be an important independent factor in the development of oropharyngal cancer.

Using the data collected in the prospective Lung Health Study (556, 557), which had enrolled 5,887 adult smokers in a clinical trial, MURRAY *et al.* (328) examined the relationship between menthol cigarette smoking and mortality from all causes, coronary heart disease, cardiovascular disease and lung cancer. A cohort of well characterized smokers had been followed over 14 years. Comparing self-reported users of mentholated *vs.* non-mentholated cigarettes, the hazard ratios due to smoking menthol cigarettes were not significant for all causes of smoking related mortality (0.997; 95% CI = 0.83–1.20), coronary heart mortality (1.31; 95% CI = 0.77–2.22), cardiovascular mortality (1.03; 95% CI = 0.70–1.52), and lung cancer mortality (0.96; 95% CI = 0.70–1.32). The authors concluded that there was no evidence of menthol in cigarettes increasing the hazards of smoking.

SCANLON *et al.* (548) compared in a prospective analysis the decline in lung function among smokers, ex-smokers and non-smokers, who were participants in the multicenter Lung Health Study (556, 557). The decline in lung function correlated with individual smoking habits and smoking history while an additional effect of cigarette mentholation was not observed.

According to the study of PLETCHER *et al.* (549) menthol and non-menthol cigarette use was equally associated with the development of atherosclerosis and decline of pulmonary function. This was in line with the findings of SCANLON *et al.* (548) concerning pulmonary function.

In 2012, VOZORIS (558) published a population-based study investigating the effects of smoking mentholated cigarettes on cardiovascular and pulmonary diseases. A total of 5,167 current smokers were studied between 2001 and 2008, of which 74.4% were regular mentholated cigarette consumers and 24.6% non-mentholated cigarette users. Mentholated cigarettes were found to increase significantly the risk of stroke compared to non-mentholated cigarettes, in particu-

lar in women and non-Afro-american smokers. There were no significant associations between smoking mentholated cigarettes and hypertension, myocardial infarction, congestive heart failure or COPD. Without a plausible explanation for his findings VOZORIS suggested further research on the subject.

Reviewing the relevant published literature (not including the paper of VOZORIS) concerning the effects of cigarette mentholation on smoking related diseases, WERLEY *et al.* (314) concluded that the inclusion of menthol in cigarettes did not increase puff volume or puff number, and had little or no effect on heart rate, blood pressure, uptake of carbon monoxide, "tar" intake or blood cotinine concentration. In addition, mentholation did not increase lung cancer risk and could not explain the higher risk for lung cancer in Afro-american male smokers.

HECK (267) concluded from these studies: "*The body of available epidemiological evidence to date provides a substantial basis for a conclusion that the risks for the development of cancers and other diseases associated with smoking of menthol cigarettes are not different, qualitatively or quantitatively, than those associated with non-mentholated cigarettes. Other authors and commentators* [(314, 559, 560)] *have previously come to similar conclusions, and all of the most recent studies…* [(328, 546, 549)] *strengthen those prior judgments. Two of these reports extend the body of evidence that the risks accompanying the smoking of menthol cigarettes are similar in magnitude to those of non-mentholated cigarettes to include data on overall mortality as well as cardiovascular and respiratory disease.*"

The conclusions of HECK (267) concerning the absence of additional health risks for smokers due to cigarette mentholation were confirmed by the Menthol Report of ALTRIA CLIENT SERVICES (280) and the U.S. Industry Menthol Report (338). The same set of studies was used in all three evaluations.

Very recently, the TOBACCO PRODUCTS SCIENTIFIC ADVISORY COMMITTEE (TPSAC) of the Center for Tobacco Products of the Food and Drug Administration (FDA) issued an extensive report "Menthol Cigarettes and Public Health: Review of the Scientific Evidence and Recommendations". After evaluating the available epidemiological studies the TPSAC concluded (339, on page 208):

"*Overall, the epidemiological studies indicate comparable risks for a number of cigarette-caused diseases in smokers of menthol compared to non-menthol cigarettes. The point estimates are largely centered around unity. Several limitations of these studies need to be noted in interpreting the findings. The extent of information on smoking of menthol cigarettes was variable and complete across the full smoking history only in one of the case-control studies. Random misclassification of menthol smoking would tend to bias estimates of the comparative risk of smoking menthol cigarettes towards unity, regardless of whether there was a "true" increase or decrease in risk for menthol cigarette smokers. Additionally, many of the studies, particularly those on cancer risk, were carried out several decades previously. Consequently, given historical patterns of menthol cigarette use, there would be few participants in these studies who had smoked menthol cigarettes across their full smoking history. Finally, the studies generally have relatively small numbers of participants. However, even with the relatively modest sample sizes of some of the studies, the point estimates do not provide any consistent indication of increased risk.*"

The TOBACCO PRODUCTS SCIENTIFIC ADVISORY COMMITTEE'S synthesis of the epidemiological evidence reads as follows (339, on page 210):

The evidence is insufficient to conclude that smokers of menthol cigarettes face a different risk of tobacco-caused diseases than smokers of non-menthol cigarettes. … Available epidemiologic data do not demonstrate increased disease risk in people, but the data are largely limited to lung cancer. The hypothesis that menthol cigarette smoking increases the risk of cardiovascular disease is biologically plausible and needs to be investigated."

Recently, HOFFMAN (561) of the U.S. FDA Center for Tobacco Products reviewed the health effects of mentholated cigarettes. Data from 89 publications were evaluated concerning possible effects of mainstream smoke, generated from mentholated cigarettes, on biomarkers of tobacco exposure, on respiratory patterns, cardiovascular function, allergic reactions and, last not least, on smoking related diseases. Based on the data reviewed, HOFFMAN concluded that menthol cigarettes did not generally appear to be more harmful than non-menthol cigarettes. Both cigarettes produced significant negative effects on health outcome, including respiratory disease, cardiovascular events and cancer. However, there was some indication that menthol cigarettes may result in more serious acute cardiovascular events. In addition, there may be subgroups of smokers, which were more or less sensitive to the health effects of cigarette smoking. HOFFMAN did not offer an explanation of the effect of menthol cigarettes on acute cardiovascular events.

KABAT *et al.*(562) examined in 2012 the ecologic association between the sales of mentholated cigarettes in the U.S. in the period 1950–2007, the preference of consuming mentholated cigarettes by race and sex, and the incidence rates of tobacco related lung cancer, squamous cell carcinoma of the esophagus, oropharyngeal cancer, and laryngeal cancer. Total sales of mentholated cigarettes increased from about 3% market share in 1950 to slightly less than 30% in 1980 and remained fairly stable thereafter. Additional data showed consistently that, compared to white smokers, Afro-american smokers favored mentholated cigarettes by a roughly 3-fold margin. The authors documented dramatic changes in the incidence of the four tobacco related cancers by race and sex over the 35-year period investigated but none of the observed trends was consistent with a strong independent effect of menthol used as a cigarette additive. They stated: "*The evidence presented here, which is inconsistent with a major effect of menthol in cigarettes as a cause of the upper aerodigestive tract cancers, brings us no closer to understanding the reason for extreme racial disparities in these cancers.*"

6. OPINIONATED REVIEWS

Literature review is becoming an increasingly important tool for summarizing scientific evidence in support of politicians' and administrators' decisions on health issues.

These reviews depend on the published literature. However, as pointed out by SONG *et al.* (563), the number of studies included in a literature review may be less than the number of all relevant studies conducted on a subject. If the studies or publications missing from a review have results that are systematically different from those included, bias or systematic error will occur.

Obviously, conducting a serious scientific discussion of the effects of the additives used in cigarette manufacturing is quite difficult and often not desired by the anti-tobacco health community as shown by the following examples of opinionated reviews:

In 2001, FOWLES and NOITON (564) prepared a report titled "Chemical factors influencing the addictiveness and attractiveness of cigarettes in New Zealand" on request by the New Zealand Ministry of Health. The report is divided in several chapters, dealing with the delivery of nicotine to, and its absorption by, smokers; the nicotine content of cigarettes; the slowing of cigarette burn rates; chemicals that produce pH changes in cigarette smoke; additives alleged to act as bronchodilators, such as cocoa, coffee extract and licorice; compounds in cigarette smoke that may influence addiction, such as acetaldehyde and levulinic acid; and the tobacco specific *N*-nitrosamines. Among the substances possibly influencing the attractiveness of cigarettes sugars, licorice, vanilla and coumarine derivatives are listed and evaluated. One of the chapters deals with additives assumed to reduce the sensory irritation by tobacco smoke and to numb peripheral nerves. Eugenol, menthol and humectants are claimed to be agents of this type. Masking the irritation and odor of cigarette sidestream smoke by additives is also discussed.

In their report on the effects of cigarette ingredients FOWLES and NOITON follow without any critique the hypotheses and speculations of BATES *et al.* (17) and CONNOLLY *et al.* (565) claiming that additives increase the attractiveness and addictiveness of cigarettes. The possible contribution of the tobacco specific *N*-nitrosamines to the addictive potential of cigarette is one of the weirdest speculations of FOWLES and NOITON.

In the fifty-three references in the report of FOWLES and NOITON studies on additives by scientists of the tobacco industry are ignored as are publications not supporting the speculative statements of the authors. Their report is neither well balanced nor scientifically correct. FOWLES and NOITON selected the scientific literature available up to 2001 on tobacco additives and the effects of nicotine in smokers in support of their request to develop regulatory schemes for assessing the addictiveness and attractiveness of tobacco products, especially cigarettes. The report contains a proposal for a regulatory framework for additives in cigarettes.

Another example of a tendentious discussion of a cigarette additive is the publication of KEITHLY *et al.* (566), titled "Industry research on the use and effects of levulinic acid: A case study in cigarette additives". Levulinic acid (4-oxopentanoic acid) is a natural tobacco component present in tobacco and tobacco smoke (567–569). According to KEITHLY *et al.* this acid and its nicotine salt were used by U.S. cigarette manufacturers as cigarette ingredients. Based on recent information submitted by the European tobacco industry to official authorities in the EU Member States, levulinic acid and levulinates are no longer used as additives in cigarette manufacturing (92). The acid was considered in 1966 by the R.J. Reynolds Tobacco Co. as a tobacco additive for improving the taste of air cured tobacco and smoothing out stemmy taste, especially of reconstituted tobacco. This trait of levulinic acid led to a patent in 1989 (570).

The complexity of the interaction of nicotine and levulinic acid in influencing the taste and smell of smoke and the impact of levulinic acid on cigarette smoke composition and mainstream "smoke pH", and on smoke toxicity were investigated intensively, especially by scientists of the R.J. Reynolds Tobacco Co. No studies or data were published in peer reviewed journals. However, more than 1,000 different documents concerning "levulinic acid" are publicly available today in the R.J.R. databank (228). These documents consist of complete study reports, interim reports, study protocols as well as inter-office messages - all of different quality and value. Obviously, in addition to valuable information these documents also contain the personal hypotheses, expectations and intentions of the involved scientists, mingled with misleading ideas and bold data interpretation. Therefore, the evaluation and rating of the contents of these documents should be done with sufficient expertise, great caution and appropriate open-mindedness.

KEITHLY *et al.* reviewed and evaluated these internal documents in a rather crude approach. The authors claimed that levulinic acid was used by the industry to increase nicotine yields in smoke while enhancing the perception of smoothness and mildness. They also alleged that the addition of levulinic acid reduced the pH of cigarette mainstream smoke and desensitized the upper respiratory tract, increasing the potential of cigarette smoke to be inhaled deeper into the lungs. The acid might also enhance the binding of nicotine to neurons in the central nervous system that ordinarily were not responsive to nicotine. All the items above would enhance the addictiveness of smoking. Observed changes in cigarette mainstream and sidestream smoke composition might produce increased health risks for smokers and non-smokers.

The interpretation of data and the conclusions drawn from these internal industry documents by KEITHLY *et al.* are an unwitting or intentional misunderstanding of the purpose of additives in cigarette manufacturing. As mentioned earlier in this paper, reducing the harshness of cigarette smoke is one of the legitimate reasons for using additives in the production of U.S. blended cigarettes. The reduction of cigarette mainstream "smoke pH" is a logical consequence of adding the volatile compound, levulinic acid, to tobacco. None of the internal documents of the R.J. Reynolds Tobacco Co. are an indication, much less proof, for the assumptions of KEITHLY *et al.* that cigarette manufacturers manipulated the addictiveness of smoking by the addition of levulinic acid to cigarette tobacco, by influencing nicotine transfer from tobacco into smoke, by increasing nicotine uptake in smokers or by intensifying the binding of the alkaloid to nicotine receptors in the central nervous system.

In 2007, RABINOFF *et al.* (571) investigated tobacco industry documents and other sources for evidence of possible chemical and pharmacological effects of tobacco

additives. The authors stated that more than 100 of the 599 tobacco additives documented in the DOULL list (223) had the pharmacological potential to mask the odor of environmental tobacco smoke, enhance nicotine delivery, increase the addictiveness of cigarettes or cover up symptoms and illnesses associated with smoking. In their conclusions, RABINOFF *et al.* followed BATES *et al.* (17), KEITHLY *et al.* (566) and others, especially regarding the influence of additives on the addictive potential of cigarettes, without evaluating technical feasibility or biological plausibility.

The examination of the possible effects of additives by RABINOFF *et al.* (571) is based on 117 references, 75 of which are not peer reviewed internal documents of the tobacco industry. These documents are research proposals, interim reports, comments, proposed working hypotheses and final reports. The contents of this kind of documents may be influenced by personal intentions, feelings and ambitions. It is also not clear which of the findings reported in these tobacco documents influenced cigarette or brand development or cigarette manufacturing to which degree. Not one of the other 42 references in the publication of RABINOFF *et al.* is a paper on additives by scientists of the tobacco industry published in peer reviewed scientific journals. RABINOFF *et al.* ignored the scientific arguments in the publication of MÜLLER and RÖPER (146) against the statements of BATES *et al.* (17) and instead arbitrarily extracted pieces of information from a concurrent internal document of the tobacco industry (572). Likewise, publications by independent researchers, such as scientists of the Dutch Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven (132, 466, 573), were not considered.

The information concerning possible pharmacological effects of selected chemical tobacco additives presented by RABINOFF *et al.* (571) suffers from poor scientific quality and is lacking seriousness. The pure compilation of potential pharmacological effects of substances without any consideration of the amounts added to tobacco and their transfer rates into mainstream smoke, of dose-effect relationships and half-life periods in the organism, etc., allows no responsible critical evaluation of the relevance of specific ingredients in cigarettes and their realistic effects on smokers. Such kind of highhanded reflex is far from a serious discussion of scientific questions. It is a worthless exercise.

The paper of STEVENSON and PROCTOR "The Secret and Soul of Marlboro" (574), published in 2008, is of similar quality. It reports a mixture of facts together with hypotheses and speculations, all together presented as "truth". The focus of this paper is the "ammonia technology" for free-basing nicotine in tobacco smoke and, consequently, increasing the addictiveness of cigarette smoking. STEVENSON and PROCTOR ascribe the success of the Philip Morris cigarette brand Marlboro to the use of this technology. Like in the publication of RABINOFF *et al.* (571), 73 of the 97 references cited by STEVENSON and PROCTOR are internal documents of the tobacco industry. Not one of the references is a paper published by a tobacco industry scientist in a peer reviewed journal. Characteristically, a comprehensive review on the analytical methodology of tobacco and its products by GREEN and RODGMAN was not cited as published in the 1996 issue of "Recent Advances in Tobacco Science" (575), which is easily available to the scientific community, but only as the concurrent internal document of R.J. REYNOLDS TOBACCO CO. (576), which is informal and has to be dug out in the Legacy Tobacco Documents Library.

There are major papers in peer reviewed journals discussing the biological plausibility (much rather, implausibility) of increasing nicotine uptake in cigarette smokers by means of "ammonia technology" (129, 132). None of these essential publications was mentioned by STEVENSON and PROCTOR (574).

In 2008, the DANISH CANCER SOCIETY (577) prepared a report on the basis of "available literature". Of this 424-page report only a short review is available in English. Besides the hypotheses, statements and speculations on the health effects of tobacco additives, which are found in the publications discussed above, the effects of other tobacco additives, such as acetophenone, benzylalkohol, organic acids, lilanool and terpenes, were speculated about. Based on what is revealed in the abstract the report of the DANISH CANCER SOCIETY cannot be regarded as a scientific paper. The speculations and statements in the document are simply intended to support the need of regulating, restricting and prohibiting the use of additives in tobacco product manufacturing because - according to the Society - they contribute to tobacco addiction.

KAHNERT *et al.* (578) of the German Cancer Research Center (DKFZ) published in 2012 a review of the effects of menthol as a tobacco additive. This paper is a publication aiming at the political goal of regulating or prohibiting tobacco use and not an unbiased scientific discussion whether or not there is an effect of menthol as tobacco additive on the toxicity of tobacco products and the health risks of tobacco consumers. Due to the one-sided selection of published studies the paper leads to false and scientifically incorrect conclusions.

In the 4th Report on the scientific basis of tobacco product regulation of the WHO STUDY GROUP ON TOBACCO PRODUCT REGULATION (TobReg), published in 2012 (116), tobacco companies, especially cigarette manufacturers, were accused of designing and manufacturing products with increased dependence (addictive) potential and attractiveness. This was alleged to be achieved by using menthol and substances like levulinic acid, urea, acetaldehyde and acetaldehyde releasing compounds, chocolate, and others. It was concluded from evaluating (parts of the open) scientific literature and formerly unpublished documents of the tobacco industry (Bates collection) that "*tobacco products are designed and manufactured to increase their dependence potential. ... The dependence potential of a product can be manipulated by designs that add ingredients with dependence-producing effects to the product and emissions, in addition to nicotine*". The list of references included in the TobReg Report shows only references, which support the view of the group and the WHO, resp. None of the studies and critiques, concerning the influence of tobacco additives on addictiveness, which were published in peer reviewed journals and are not in line with the opinion of the group, are mentioned. Therefore, this part of the TobReg Report cannot be accepted as a scientific basis for tobacco product regulation even though reputable scientists were members of the Study Group.

7. CONCLUDING REMARK

In summary, the results of the scientific work evaluated in this review show that tobacco additives have only occasional and limited effects on cigarette mainstream smoke composition, which are almost never reflected in toxicological *in vitro* assays or *in vivo* studies, and do not confirm the assumption that the additives used in cigarette manufacturing increase the risk of smokers for any cancers, chronic obstructive lung disease or cardiovascular diseases. It is unlikely that nicotine availability or nicotine addictiveness are enhanced and that additives seduce adolescents to smoke or reduce the effectivity of smoking cessation measures. For certain consumers aromatic additives may increase the attractiveness of a specific cigarette brand compared to other brands without affecting cigarette consumption in general.

In his response to a request from the U.S. National Cancer Institute concerning research opportunities related to establishing standards for tobacco products under the U.S. Family Smoking Prevention and Tobacco Control Act, HECHT (579) stated recently: "*Ingredients, which are additives, require less attention than nicotine and harmful or potentially harmful constituents*" (of tobacco and tobacco smoke). We believe that this statement describes additives in comparison to other tobacco and tobacco smoke components in a correct way and puts them in the right place regarding their importance for the health of tobacco consumers.

ACKNOWLEDGMENT

The authors wish to express their great appreciation for Dr. Barbara Boenke´s exceptional involvement in the composition of this review, particularly for her support in identifying and retrieving numerous scientific documents and publications. The authors would also like to thank Dr. Anja Thielen for many helpful discussions during the preparation of the manuscript.

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