# ADDICTIVENESS AND ATTRACTIVENESS OF TOBACCO ADDITIVES: ANALYTICAL AND TOXICOLOGICAL CHARACTERISATION

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Herewith I certify that I have prepared and written my thesis independently and that I have not used any sources and aids other than those indicated by me.

## Abstract

*"The only way to avoid a smoking-related risk is not to smoke"* (Baker, 2006).

Tobacco smoke consists of a multitude of harmful substances many of them known carcinogens. In addition, the tobacco alkaloid nicotine is a highly addictive compound, thus attempts to quit smoking in most cases fail. Therefore the best way to prevent smoking related diseases is to prevent people from getting started to smoke. The revision of the European Tobacco Product Directive 2014/40/EU (EU, 2014) is targeted on this approach. Concerns raised that tobacco additives could be used to facilitate the addictiveness and attractiveness of the product. And by that means to attract especially young people and entrap them to become addicted smokers. Under Article 6 of the relaunched Directive manufacturers and importers are required to submit data that confirm that the ingredients used do not

- increase the toxicity or the addictive potency of the product,
- result in a characteristic flavour,
- or facilitate inhalation or nicotine uptake.

To provide answers to this complex question, of whether tobacco additives can potentially enhance the effect of the inherently harmful and addictive consumer product tobacco, an on-line coupled oxidative pyrolysis technique was established and coupled to gas chromatography-mass spectrometry (GC/MS). By applying this instrumental tool, 19 different tobacco additives like raw cane sugar, liquorice or cocoa as well as additive-free tobacco were screened for their thermal decomposition products after pyrolysis. In the present work 72 different compounds with toxic potential were detected. Among these compounds, vinyl acrylate, fumaronitrile, methacrylic anhydride, isobutyric anhydride and 3-buten-2-ol were exclusively detectable upon pyrolysis of tobacco additives. By contrast, these toxicants revealed undetectable in tobacco smoke itself. The most frequently formed pyrolysis products were formaldehyde and acetaldehyde. A semi-quantitative approach used to look into the formation of polycyclic aromatic hydrocarbons during pyrolysis of cocoa and/or tobacco could demonstrate that the addition of cocoa to tobacco is unlikely to significantly alter the relative amounts of these highly carcinogenic compounds in the smoke of cigarettes refined with this flavour.

Article 7 of the Directive regulates the prohibition of additives that create a characteristic flavour in cigarettes and in roll-your-own tobacco. It remains unclear how to reliably measure such properties. A powerful chemical approach based on headspace (HS) solid phase microextraction (SPME) coupled to GC/MS was elaborated to identify chemical markers for complex fruity flavours. These marker substances can now be further used

to assign manufacturers' data on tobacco additives that have been employed to flavour their products. Considered as an exemplary case, the chemical marker substances for strawberry flavour were identified applying this method. This work resulted in a list of 11 different chemical compounds that appropriately indicate the presence of strawberry flavour. Overall the established approach provides a fast analysis of characteristic flavours used in tobacco products. For further clarification additional analytical means including sensory tests would be mandatory.

Mentholated cigarettes are part of the regulation of characteristic flavouring of tobacco products. As menthol is biologically active the issue was to be addressed whether tiny amounts of this compound in cigarettes that remain below the sensory threshold would still be sufficient to induce a physiological response, that is, a cooling sensation in the bronchial epithelia, and thus may facilitate deeper inhalation of tobacco smoke in the lungs of consumers. To this end, analytical methods were to be developed that would allow reliable quantification of the menthol levels in cigarettes. An analytical GC/MS based method has thus been established and validated to quantify this compound in tobacco products. Based on this we found that only some mentholated cigarettes contained menthol levels high enough to create a characteristic flavour. On the other hand, menthol contents of American blend, additive-free and light cigarettes remained below this sensory threshold. In these cases, however, the issue had to be addressed whether or not even such sub-sensory levels of menthol would still be capable of activating menthol responsive cold receptors. For that reason, a bioassay has been established suited to determine the minimum menthol content needed to activate the transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) protein. The experimental data show that menthol levels in cigarettes above 4 µg/cigarette are well be able to activate the TRPM8 cold receptor although they would not necessarily fall under the tobacco regulation of characteristic flavourings. The non-mentholated cigarettes investigated in our studies, however, did not exceed this value of 4 µg/cigarette. Yet it is known from the literature that there are non-mentholated cigarettes on the market with menthol contents certainly high enough to activate this TRPM8 receptor. Such cigarettes might therefore facilitate deeper inhalation, an increased nicotine uptake during smoking and, ultimately, an amplified addictiveness of the respective product.

For the assessment of chiral compounds that might also be applied as tobacco additives (i.e., menthol), the distinction between the individual enantiomers would be crucial. Since enantiomers usually differ in their physiological properties, their ability to activate the TRPM8 receptor or their kind of flavour perception could be different as well. As a most welcome side effect enantioselective analysis would also allow to identify the origin of

the respective compound, and to decide whether it would be natural or synthetic. For that reason, an enantioselective (Es) GC/MS method has been developed to enable the distinction between enantiomers in selected terpenoids present in strawberry flavourings. The established method would also be suitable to identify the enantiomeric composition of menthol in tobacco products. This latter aspect would be of particular interest if menthol addition to tobacco products will be restricted by law. Since menthol occurs naturally in the leaves of the tobacco plant, an Es-GC/MS analysis could serve as authenticity proof for the origin of this compound in cigarettes.

## Zusammenfassung

*„Der einzige Weg zur Vermeidung rauch-induzierter Risiken ist nicht zu rauchen“* (Baker, 2006).

Tabakrauch besteht aus einer Vielzahl gesundheitsschädlicher Verbindungen, darunter viele bekannte Kanzerogene. Darüber hinaus handelt es sich bei dem Tabak-Alkaloid Nikotin um einen sehr starken Suchtstoff, wodurch die meisten Versuche mit dem Rauchen aufzuhören missglücken. Von daher ist der beste Weg den Folgen des Tabakkonsums vorzubeugen, den Einstieg zum Tabakkonsum vorzubeugen. Die Neufassung der Tabak-Produktrichtlinie 2014/40/EU (EU, 2014) stützt sich auf diesen Gedanken. Bedenken kamen auf, dass Tabakzusatzstoffe dazu verwendet werden könnten, eine suchtverstärkende Wirkung oder eine Attraktivitätssteigerung des Tabakprodukts zu erzielen. Auf diese Weise würden besonders Jugendliche dazu verführt werden, mit dem Rauchen anzufangen. Unter Artikel 6 der Richtlinie werden Hersteller und Importeure zur Bereitstellung von Daten verpflichtet, die zeigen, dass die verwendeten Zusatzstoffe nicht dazu beitragen,

- die Toxizität oder die Attraktivität des Produktes zu erhöhen,
- zu einem charakteristischen Aroma führen,
- oder die Inhalation sowie die Nikotinaufnahme zu erhöhen.

Zur Klärung der komplexen Fragestellung, ob Tabakzusatzstoffe potentiell dazu in der Lage sind, die Wirkung des an sich bereits gesundheitsschädlichen und suchterzeugenden Verbraucherprodukts Tabak zu steigern, wurde eine Methode zur oxidativen Pyrolyse, on-line gekoppelt an die Gaschromatographie mit anschließender Massenspektroskopie (GC/MS), entwickelt. Mit dieser Methode wurden 19 verschiedene Tabakzusatzstoffe wie Rohrohrzucker, Süßholz, Kakao, etc., sowie zusatzstofffreier Tabak hinsichtlich ihrer thermischen Zerfallsprodukte nach der Pyrolyse analysiert. In der vorliegenden Arbeit konnten 72 verschiedene Substanzen mit toxischem Potential detektiert werden. Darunter befanden sich Stoffe wie Vinylacrylate, Fumaronitril, Methacrylsäureanhydrid, Isobuttersäureanhydrid und 3-Buten-2-ol, die ausschließlich während der Pyrolyse von Tabakzusatzstoffen gebildet werden. Die am regelhaftesten durch Pyrolyse gebildeten Substanzen waren Formaldehyd und Acetaldehyd. Weiterhin wurde in einem semi-quantitativen Ansatz die Bildung von Polycyclischen Aromatischen Kohlenwasserstoffen nach der Pyrolyse von Kakao und/oder Tabak untersucht. Hierbei konnte gezeigt werden, dass die Zugabe von Kakao zu dem Tabak die relativen Mengen der untersuchten Verbindungen nicht beeinflusst.

Artikel 7 der Richtlinie reguliert das Verbot von Zusatzstoffen die zur Bildung von charakteristischen Aromen in Zigaretten und Tabak zum Selbstdrehen beitragen. Wie diese Eigenschaften gemessen werden sollen bleibt unklar. Aus diesem Anlass wurde im Rahmen der vorliegenden Arbeit eine Untersuchungsmethode entwickelt, die es ermöglichen soll, basierend auf Headspace (HS) Festphasen Microextraktion (SPME), gekoppelt an eine GC/MS-Analytik, chemische Marker Substanzen für komplexe fruchtige Aromen zu identifizieren. Diese chemischen Marker könnten dann zukünftig genutzt werden, um die von der Tabakindustrie übermittelten Daten zu den verwendeten Zusatzstoffen zu überprüfen und die Präsenz charakteristischer Aromen nachzuweisen. Mittels der etablierten Methode wurden exemplarisch die chemischen Marker für das Erdbeeraroma definiert. Dabei ergab sich eine Gruppe von 11 verschiedenen chemischen Verbindungen, die für die Verwendung als Erdbeeraroma-Marker geeignet sind. Dieser Ansatz bietet somit eine Option zur analytischen Prüfung der Anwesenheit von charakteristischen Aromen in Tabakerzeugnissen. Ergänzende Verfahren, beispielsweise sensorische Tests, könnten dann zur abschließenden Verifikation eingesetzt werden.

Menthol-Zigaretten fallen ebenfalls unter die Regulierung charakteristischer Aromen in Tabakerzeugnissen. Da es sich bei Menthol um eine biologisch aktive Substanz handelt, muss untersucht werden, ob auch Menthol-Gehalte unterhalb der sensorischen Wahrnehmungsgrenze ausreichend sind, um einen kühlenden Effekt in Bronchialzellen erzeugen zu können, welcher dann zur erleichterten Inhalation von Tabakrauch beitragen würde. Für diese Fragestellung werden analytische Methoden benötigt, die es erlauben, den Menthol-Gehalt in Zigaretten und Zigarettenrauch zu bestimmen. Im Rahmen dieser Arbeit wurde eine GC/MS-basierte Methode erarbeitet und validiert, um Menthol in Tabakprodukten zu quantifizieren. Bei einigen der Menthol-Zigaretten konnte ein ausreichend hoher Menthol-Gehalt nachgewiesen werden, der für die Ausprägung eines charakteristischen Aromas im Produkt maßgeblich war. Die Menthol-Gehalte der untersuchten „American Blend“-, zusatzstofffreien und „Light“-Zigaretten lagen dagegen unterhalb der Wahrnehmungsgrenze. Daher ergab sich die anschließende Fragestellung, ob ein solch geringer Menthol-Gehalt in Zigaretten ausreichend ist, um den Menthol-Kälterezeptor zu aktivieren und damit zur Sensorik eines kühlenden Effekts während des Rauchens beizutragen. Aus diesem Grund wurde im Rahmen dieser Arbeit ein Bioassay etabliert, der es erlaubt, die minimale Menge an Menthol zu bestimmen, die benötigt wird, um das Transient Receptor Potential Cation Channel Subfamily M (Melastatin) Member 8 (TRPM8) Protein zu aktivieren. Die daraus resultierenden Daten zeigen, dass Menthol-Gehalte ab 4 µg/Zigarette zwar nicht zwangsläufig unter die Definition der charakteristischen Aromen fallen, aber dennoch in der Lage sind, den

TRPM8 zu aktivieren. Keine der hier untersuchten mentholfreien Zigaretten überschritt den Wert von 4 µg/Zigarette. Allerdings geht aus der Literatur hervor, dass angeblich mentholfreie Zigaretten auf dem Markt erhältlich sind, die ausreichend Menthol enthalten, um den TRPM8 zu aktivieren. Solche Zigaretten könnten ebenfalls die tiefere Inhalation des Tabakrauchs und damit die Nikotinaufnahme erleichtern.

Für die Beurteilung von chiralen Verbindungen, die als Tabakzusatzstoffe genutzt werden (z.B. Menthol), kann die Unterscheidung zwischen den Enantiomeren wichtig werden. Die individuellen Enantiomere einer chiralen Verbindung können sich prinzipiell in ihren physiologischen Eigenschaften, beispielsweise in ihrer Fähigkeit, den TRPM8 zu aktivieren, oder in ihrer Ausprägung einer speziellen Geruchsnote, unterscheiden. Zusätzlich eignet sich eine enantioselektive Analytik auch dafür, Schlüsse über die Herkunft (natürlich oder synthetisch?) einer chiralen Substanz zu ziehen. Aus diesem Grund wurde eine enantioselektive (Es) GC/MS-Methode entwickelt, die es ermöglicht, eine Unterscheidung zwischen den Enantiomeren einiger Terpene des Erdbeeraromas vorzunehmen. Diese Methode ließe sich dann auch zur Trennung der Enantiomeren von Menthol, das Tabakprodukten zugesetzt wird, verwenden. Eine solche Möglichkeit wäre von großem Nutzen, wenn Menthol als Zusatzstoff verboten wird, da die Verbindung auch natürlicherweise in den Blättern der Tabakpflanze vorkommt. Mithilfe der hier entwickelten Es-GC/MS-Methode könnten somit Rückschlüsse über die Herkunft des Menthols in Zigaretten gezogen werden.

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# Content

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## Abbreviations

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| cDNA | complementary Deoxyribonucleic Acid |
| CFP-nuc | Cyan Fluorescent Protein-Nuclear Marker |
| CIS | Cooled Injection System |
| CMR | Carcinogenic Mutagenic Reprotoxic |
| COPD | Chronic Obstructive Pulmonary Disease |
| DKFZ | German Cancer Research Centre |
| EPC | Electronic Pneumatics Control |
| Es-GC/MS | Enantioselective GC/MS |
| EU-CEG | EU Common Entry Gate |
| FCTC | Framework Convention on Tobacco Control |
| FDA | Food and Drug Administration |
| FEMA | Flavour and Extract Manufacturers Association |
| GC | Gas chromatograph(y) |
| GRAS | General Recognized As Safe |
| HEK | Human Embryonic Kidney (cells) |
| HS | Headspace |
| IAP | Independent Advisory Penal |
| IARC | International Agency on Cancer Research |
| ISO | International Organization for Standardization |
| JATC | Joint Action on Tobacco Control |
| MAO | Monoamine Oxidase |
| MS | Mass-Spectrometer (Spectrometry) |
| nACh | nicotinic acetylcholine (receptor) |
| OAV | Odor Active Value |

| | |
|---|---|
| PAHs | Polycyclic Aromatic Hydrocarbons |
| py-GC/MS | Pyrolysis GC/MS |
| QDA | Quantitative Descriptive Analysis |
| RYO | roll-your-own |
| SCENIHR | Scientific Committee on Emerging and Newly Identified Health Risks |
| SIM | Selected Ion Monitoring |
| SPME | Solid Phase Microextraction |
| TabakerzG | Tabakerzeugnisgesetz |
| TabakerzV | Tabakerzeugnisverordnung |
| TCS | Tobacco Control Scale |
| TDU | Thermal Desorption Unit |
| TIC | Total Ion Chromatogram |
| TPD | Tobacco Products Directive |
| TRP | Transient Receptor Potential |
| TRPA | Transient Receptor Potential Ankyrin |
| TRPC | Transient Receptor Potential Canonical |
| TRPM | Transient Receptor Potential Melastatin |
| TRPN | Transient Receptor Potential Drosophila NOMPC |
| TRPP | Transient Receptor Potential Polycystin |
| TRPRL | Transient Receptor Potential Mucolipin |
| TRPV | Transient Receptor Potential Vanilloid |
| WHO | World Health Organisation |

# 1 Introduction

The epidemic spreading of tobacco consumption in Europe already started in the 16th century, when Spanish sailors brought the plant (Figure 1-1) from the “new world” to the “old world”. In adaption to the use of tobacco for mystic, social and medical rituals by the American natives, in the following tobacco was also extensively promoted as medical plant by Europeans (Sanchez-Ramos, 2020). The discovery of deleterious health effects needed till the mid of the 20th century, when epidemiology studies irrefutably uncovered the association between tobacco smoking and the increase in lung cancer cases (Warren and Cummings, 2013).

Its botanic name *Nicotiana tabacum* (plant family of nightshades, *Solanaceae*) was assigned in honour of Jean Nicot who propagated tobacco as a medical plant in France. He treated the recurring headache of Queen Caterina de’ Medici with snuff in the 16th century (Sanchez-Ramos, 2020). The word tobacco, however, traces back to tobacco leaves which were rolled to a cylinder, called Tabako, which helped indigenous people of Hispaniola (Cuba) to chase away mosquitos. Also, the pipes used to smoke tobacco by the Mexicans were called “Tabakos”, whereas the plant itself was named yetl or pycielt by American natives (KÖHLER`S ATLAS der Medizinal-Pflanzen, 1997).

**Figure 1-1: *Nicotiana tabacum L.* (from KÖHLER`S ATLAS der Medizinal-Pflanzen, 1997)**

## 1.1 Tobacco addiction

Although some positive effects of tobacco may remain undisputed, the regular use quickly leads to habituation. As consequence it is hard to quit smoking once it has been started (Sanchez-Ramos, 2020). The addictive potency of tobacco smoke is mainly attributed to the tobacco alkaloid nicotine. By activation of the nicotinic acetylcholine (nACh) receptor, neurotransmitters such as dopamine, noradrenaline, acetylcholine, vasopressin, serotonin and β-endorphin become activated in the brain (Marquardt et al., 2019). The overall effect is the enhancement of mood triggered by the mesolimbic reward system which is predominantly dependent on dopamine (Fig 1-2). Conversely, long-term (repeated) activation of the nACh receptor by agonists such as nicotine leads to the onset of neuroadaptation, e.g., receptor desensitisation or reduction of the number (density) of receptors. This effect is assumed to cause some kind of “tolerance” (habituation) against nicotine resulting in symptoms of craving and withdrawal (Benowitz, 2010). Both mood enhancement and prevention of withdrawal symptoms play an important role in nicotine addiction.

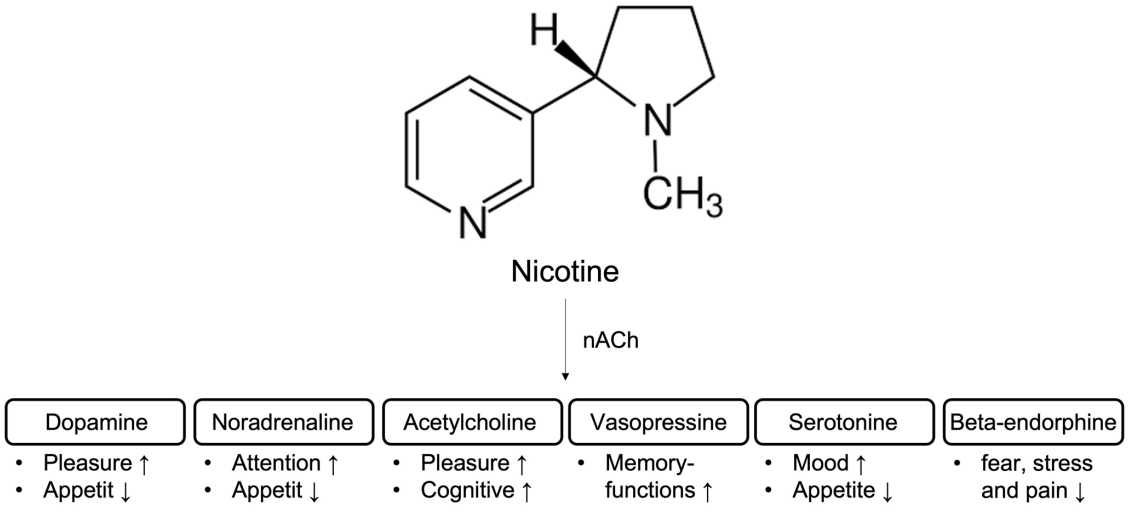


**Figure 1-2: Important neurotransmitters and their functions emerging after activation of the nACh receptor in the brain (adapted from Marquardt et al., 2019)**

On the other hand, it is assumed that some kind of reward feeling is already initiated even prior to the onset of the pharmacological effects caused by binding of nicotine to its nACh receptor. This sensory pleasure is activated immediately after drawing on a cigarette and might result somehow from the haptic experience itself. Since it is thought to contribute to the addictive potential of tobacco smoking it should not be

underestimated as important factor to be considered in the efforts towards cessation of smoking habits (Marquardt et al., 2019).

## 1.2 Cigarettes

With the beginning of the industrial production of cigarettes, starting in the late 19th century, previous tobacco products such as cigars, pipe and chewing tobacco became successively displaced. American soldiers received a weekly allowance of 50 cigarettes during World War I. After returning home most of them were already solid and addicted smokers. In the following decades, lung cancer incidences increased dramatically (Warren and Cummings, 2013). The link between smoking and lung cancer was first proposed by Fritz Licknit in 1930 (Licknit, 1930). To alleviate the growing concerns about the health effects of smoking, cigarettes with filters and lower-yield cigarettes were introduced to the market. However, it is indicated that the relative risk of smoking induced lung cancer actually increases over the years (Warren and Cummings, 2013).

The usage of filter materials that reduce the quantities of particulate matter in the smoke inhaled, also affect the sensory perception of cigarette smoke (Marquardt et al., 2019). This sensory loss and other technological advantages may have therefore contributed to the introduction and subsequent excessive use of additives in cigarette manufacturing.

## 1.3 Cigarette additives

Commercially available well-known cigarette brands consist not only of fermented tobacco, paper and filters. There are about 600 ingredients known, which are added during the manufacturing of cigarettes (Rodgman and Perfetti, 2009). These additives serve to fulfil different purposes. They are used for technological reasons, for instance glycerine or propylene glycol are added as humectants (Rodgman, 2002). To achieve brand-specific characteristics, flavourings or even complex matrixes are being used. The main role as complex matrix additives refers to the group of sugars, which need to be supplemented to replenish their loss during the drying process of the tobacco leaves (Roemer et al., 2012). Cocoa and licorice are also commonly used complex additives either as flavourings or as casing material. Casings are added to the tobacco before drying of the leaves (Simms et al., 2019) and make up between 1 and 5% by weight of the tobacco in a cigarette (Paumgartten et al., 2017). Individual flavour compounds (so-called toppings) are also used besides the complex matrices to achieve brand-specific characteristics. These toppings correspond to about 0.1% of the cigarette (Paumgartten et al., 2017). However, besides brand characteristics and technological benefits, additives can also contribute to the attractiveness, addictiveness or toxicity of cigarettes.

## 1.4 WHO Framework Convention on Tobacco Control

The global epidemics of tobacco consumption led to the adoption of the World Health Organisation (WHO) Framework Convention on Tobacco Control (FCTC) in 2003. The treaty came in force in 2005 and is now ratified by more than 190 countries. It is both an evidence-based and political agreement that aims to reduce the global tobacco use. The WHO FCTC provides reduction strategies of tobacco demand and supply. Article 9 of the WHO FCTC pledges participating countries ("parties") to measure and to regulate both contents and emissions of tobacco products. Parties did further agree on guidelines for implementation of recommendations to reduce attractiveness, addictiveness and toxicity (WHO, 2013). However, so far only the issue of attractiveness is being covered in detail to date. Parts of the guidelines were adopted by the European Commission in Directive 2014/40/EU.

## 1.5 EU Tobacco Products Directive 2014/40/EU

The European Directive on the manufacturing, presentation and sale of tobacco and related products was relaunched in April 2014 (EU, 2014). The main target of the new Directive is to reduce the attractiveness of tobacco products in particular among children and adolescents. Further, the regulation of nicotine-based electronic cigarettes was implemented for the first time. Major provisions of the Tobacco Products Directive (TPD) are:

- The prohibition of cigarettes and roll-your-own (RYO) tobacco with characterising flavours.
- No additives shall be used that enhance the toxicity, addictiveness and attractiveness of the tobacco product.
- The manufacturers have to inform the authorities about ingredients used in the manufacture of the tobacco products.
- Health relevant warnings have to be placed on the outside packaging of tobacco products. Covering 65% of the front and back of a packing unit.
- Labelling information that promotes the product must not be used on the packaging.
- Member States have to ensure the traceability of tobacco products on the market.
- Novel tobacco products shall be notified to the Member States before their implementation on the market.
- Electronic cigarettes and refill containers are regulated by this Directive as well.

In Germany the legislative provisions for the implementation of the TPD are enacted by the Tabakerzeugnisgesetz (TabakerzG, 2016) and implemented by the Tabakerzeugnisverordnung (TabakerzV, 2016).

## 1.6 Influences on the addictiveness and attractiveness of tobacco products

The addictive effect of cigarette smoke correlates with the content level and bioavailability of nicotine (Marquardt et al., 2019). The uptake of nicotine via biological membranes mainly depends on local pH-values. Since nicotine in its natural form is a weak chemical base with a p*K*a of 8.0, buccal absorption would be improved in an alkaline environment. In tobacco used for pipes and cigars where the smoke is lengthy kept in the mouth, high pH-values in the smoke will be preferred. Due to the irritating properties of alkaline tobacco smoke, however, in the case of cigarettes a lower pH-value is being preferred to promote a deeper inhalation of the smoke to the lungs (Hukkanen et al., 2005). For nicotine absorption in the lung the pH-value of the tobacco smoke is of minor relevance. Since the smoke is being distributed all-over the huge surface area of the small airways (bronchioli) and alveoli, the nicotine will be completely dissolved in the airway fluid of pH 7,4 anyway.

The pH-value in the tobacco smoke depends strongly on the contents of sugars in the unburnt tobacco. This is due to the degradation of sugars to acidic compounds whilst smoking (Talhout et al., 2006). Sugar contents of the manufactured tobacco depend on the kind of tobacco leaves used for production. For instance, *Virginia* Tobacco (mainly used for cigarette production) has a sugar content of 8-30%, whereas *Burley* tobacco (mainly used for pipe tobacco) has only 1-2% (Banožić et al., 2020). In addition, the content of sugars in the final tobacco can be modulated by different curing methods during processing of the tobacco leaves. Flue-cured tobacco is mainly used for cigarette production, whereas air-cured tobacco predominantly serves in pipes and cigars. The latter drying process employs lower temperatures that result in rather low levels of sugars. The pH-value in the smoke from air-cured tobacco has a value of 6.5 or higher (Hukkanen et al., 2005). Flue-cured tobacco, on the other hand, is dried at higher temperatures for shorter periods resulting in higher contents of sugars (Banožić et al., 2020) and thus in a lower pH-value (about 5.5-6.0) of the smoke generated (Hukkanen et al., 2005).

Besides these technological measures, pH-values in the mainstream smoke of cigarettes can also be affected by the addition of certain additives. During saucing (i.e., casing, that is, applying a pre-cutting solution or sauce to tobacco) a mixture of flavourings, (e.g.,

citrus and fruit extracts) and sugars (e.g., corn syrup, molasses or honey) is added to the tobacco. These substances are primarily used for flavouring reasons, but can be expected to lead to changes in the pH-values of the cigarette smoke and thus influence the nicotine uptake in the respiratoy tract.

## 1.7 Toxic potential of tobacco additives

Although lung cancer was a rare disease in the early 20$^{th}$ century, nowadays it is among the main cancer-related causes of death in men. Tobacco smoke is assumed to be aetiologically responsible for 85-90% of lung cancer cases (Kaminski, 2012). And even second-hand smoking increases the probability of the induction of lung cancer by 30%, when compared to people without any exposure to passive smoke (Kaminski, 2012). Based on these findings, it seems obvious that modern designed cigarettes may contribute that smoking nowadays might be even more dangerous (Warren and Cummings, 2013).

Many additives used in the manufacturing process of cigarettes are used in the food industry and are thus proven in terms of their potential toxicity. These additives are termed “Generally Recognized As Safe” (GRAS) according to the Food and Drug Administration (FDA) regulation and/or listed as safe by the Flavour and Extract Manufacturers Association (FEMA) (FDA, 2022a; FEMA, 2022). However, both the GRAS and FEMA status of a substance only provides information related to the oral and dermal routes of uptake and thus for their admission through the digestive tract or the skin barrier, but not for the inhalation route through the lungs, especially after combustion.

Among the most commonly used flavouring chemicals are menthol, 2,3-butanedione (“diacetyl”, buttery-flavour) and 2,3-pentanedione (buttery/caramel-flavour). Menthol, for instance, may lead to oxidative stress and inflammatory and barrier dysfunctions in the cells and tissues exposed (Kaur et al., 2018). In the case of diacetyl, there is evidence that this compound can cause a decrease in the respiratory function, *resp.*, capacity (Kaur et al., 2018). It probably facilitates a disease called bronchiolitis obliterans, also known as “popcorn lung”, which leads to an irreversible damage of respiratory epithelia. Other flavouring chemicals such as aldehydes can cause cytotoxicity and irritation of the cells lining the respiratory tract (Kaur et al., 2018). Furthermore, it must be taken into account that even additives that have no toxic potential by their own, whether oral, dermal or inhaled, their pyrolysis products might have though.

## 1.8 Evaluation of smoke constituents

The formation of smoke constituents from a burning cigarette depends on different conditions, such as temperature, pH-value (Torikai et al., 2004), heating rate (Vàrhegyi et al., 2009), oxygen concentration (Senneca et al., 2007) and chemical environment (Thalhout et al., 2006). The smoking of a cigarette causes different zones of heating conditions for the tobacco (Baker, 2006). On the tip of the cigarette temperatures can rise up to 950°C during the puff. Between the puffs the temperature of the cigarette tip still remains high with temperatures of about 350°C. The burning cone is also called combustion zone, because oxygen levels are also high at the cigarette tip. Here carbohydrates (sugars) of the cigarette matrix undergo oxidation, thereby leading to the formation of simple gases such as carbon dioxide, carbon monoxide and hydrogen. By contrast, combustion in the adjacent pyrolysis/distillation zone occurs under $O_2$-depleted conditions and with lower temperatures. In this area ("pyrolysis zone") most of the smoke ingredients will be formed. Further down of the pyrolysis zone in the adjoining main tobacco roll the smoke cools rapidly down while passing this area. Here, most of the aerosol particles are formed under condensation conditions (Fig. 1-3) (Baker, 2006).

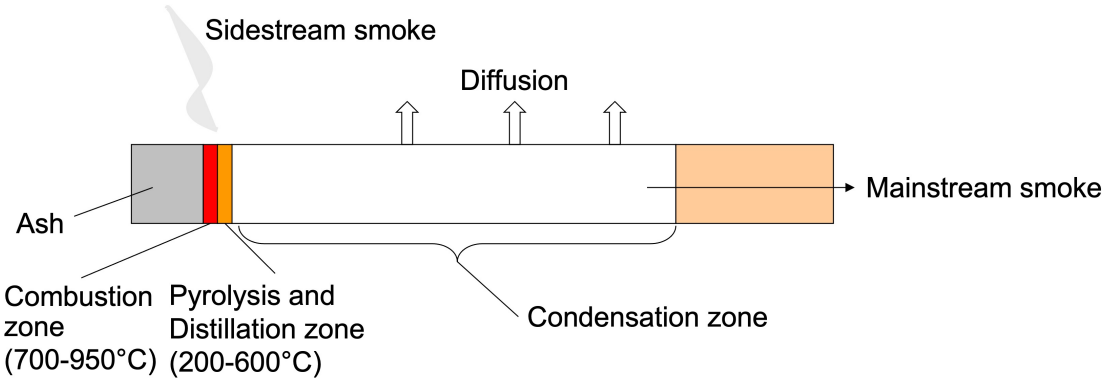


**Figure 1-3: Heating zones (conditions) in a burning cigarette (adopted from Baker, 2006)**

According to the Surgeon General´s report on smoking and health more than 7000 chemicals can be detected in cigarette smoke (US, 2010). Many of these substances are classified in accordance to the International Agency on Cancer Research (IARC) as proven (1), probably (2A) and possibly (2B) carcinogenic in man (Paumgartten et al., 2017). At least 69 of them belong to the group of human carcinogens. Besides such compounds many other toxicants were also found (US, 2010). In the literature 44 of the toxicologically relevant smoke ingredients are discussed to be relevant in the aetiology of smoking related diseases (Baker et al., 2004). These substances are also called "Hoffmann analytes" as they were first mentioned by Dietrich Hoffmann while working at the American Health Foundation in the mid 1980s (Baker et al., 2004).

In accordance to the Directive 2014/40/EU, 15 additives (Table 1-1) were set on a priority list by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). These additives are under particular consideration for the enhanced reporting obligations of manufacturers and importers of cigarettes and RYO tobacco to the Member States of the European Union.

**Table 1-1: Priority list of tobacco additives (adapted from Simms et al., 2019)**

| ***Tobacco Additives*** | | |
|---|---|---|
| *Cocoa* | *Maltol* | *Diacetyl* |
| *Carob Bean* | *Guaiacol* | *Titanium Dioxide* |
| *Fig* | *Guar Gum* | *Liquorice* |
| *Fenugreek* | *Propylene Glycol* | *Sorbitol* |
| *Menthol* | *Glycerol* | *Geraniol* |

By the Implementation Decision (EU) 2016/787 (European Commission, 2016a) comprehensive studies shall examine whether these additives

- contribute to, or increase, the toxicological or addictive behaviour of the product,
- result in a characterising flavour,
- facilitate inhalations or nicotine uptake, or
- lead to the formation or increase of substances with Carcinogenic Mutagenic Reprotoxic (CMR) properties in the product at a significant or measurable degree.

Whilst smoking, and similar to the tobacco itself, all additives become more or less chemically modified alongside the complex and different heating zones in the cigarette. Therefore the assessment of the additives must be performed exactly under these special smoking conditions.

### 1.8.1 Smoking machine measurements

With the attempt to develop harm reduced cigarettes in the early 1950s, methods were needed to measure the nicotine and tar contents of cigarette smoke. By that time smoking machine measurements were introduced and frequently used.

Smoking machines are meant to simulate the smoking behaviour of consumers in a systematic way and enable to compare tar and nicotine yields in the cigarette smoke. The volume, duration, frequency and the length of the cigarette smoking process can be adapted. When it comes to modern design cigarettes, another important parameter would be the blockage of the ventilation holes in the cigarette filter (Marian et al., 2009).

Most commonly used machine smoking protocols are the International Organization for Standardization (ISO) (ISO 3308, 2012) protocol and the Canadian Protocol (Health Canada, 2000). The differences of these two methods are shown in table 1-2.

**Table 1-2: Smoking machine parameters for the ISO and Canadian method.**

| Parameter | ***ISO*** | ***Canadian*** |
|---|---|---|
| ***Puff Volume [ml]*** | *35* | *55* |
| ***Puff Duration [s]*** | *2* | *2* |
| ***Puff Frequency [s]*** | *60* | *30* |
| ***Filter blockage [%]*** | *0* | *100* |

The particular matter present in the generated smoke is usually collected on glass fibre filters, so-called Cambridge filters. The total particular matter can then be analysed by weight differences of the filters before and after smoking. The parameter “tar” means the amount of total particulate matter minus its contents of water and nicotine. Furthermore, the smoke can also be collected in a liquid trap before being subjected to chemical analytics (Marian et al., 2009).

In the meantime, however, it became clear that the available smoking machine protocols did not adequately reflect the smoking behaviour in humans (Hammond et al., 2007). For instance, there is great dispute on whether lower yield cigarettes are really less harmful than others. This doubt derived from the observation that human smoking behaviour is mainly driven by the level of nicotine uptake rather than anything else (Hammond et al., 2007). As a consequence, the lower machine-measured yield cigarettes are usually smoked more intensively than higher yield cigarettes (Hammond et al., 2007; US, 2010). Also, the assumption of consumers that lower yield cigarettes would be associated with lower health risks, may mistakenly rather contribute an overall increase in cigarette caused mortality due to delayed quitting or heightened cigarette use.

### 1.8.2 Pyrolysis measurements

Pyrolysis refers to the thermal degradation process caused under exclusion of oxygen (Chen et al., 2018). This method can be used to simulate the high temperature zones emerging in the cigarette whilst smoking. However, in the outer combustion zone of the cigarette high levels of oxygen are available that affect the degradation process. Therefore oxidative pyrolysis methods are also required to simulate the smoking process in this particular zone of the cigarette. Usually oxidative pyrolysis is carried out in external pyrolysis chambers, hence leading to the necessity to collect the generated volatile

compounds on glass fibre filters in a similar way as explained above in the case of smoking machine generated substances. In such a setup, direct and oxygen-free connection to the gas chromatography (GC) device becomes crucial, since oxygen must be strictly excluded from the analytical column and the ion source in order to avoid damage of the whole system.

This issue can be bypassed by collecting the analytes after oxidative pyrolysis in a cryotrap and by excluding the oxygen from the GC system using a backflush (Desmet et al., 2003). The complex system used for an on-line oxidative pyrolysis GC mass spectrometry (py-GC/MS) approach is illustrated in Figure 1-4.

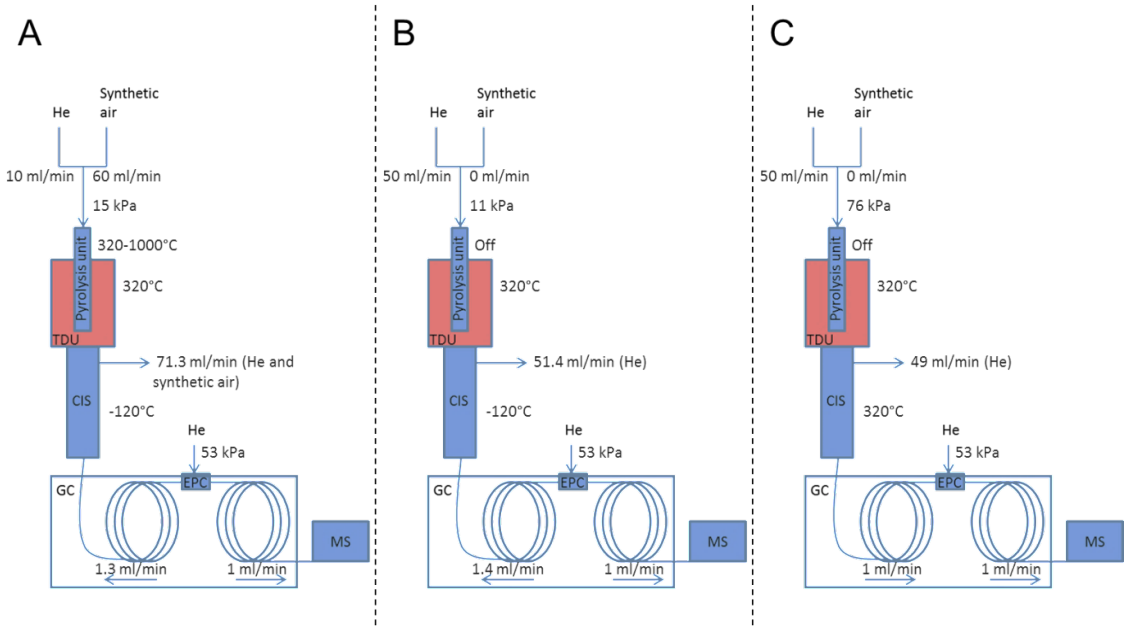


**Figure 1-4 Work flow of the oxidative py-GC/MS method (from Paschke et al., 2016).**
The backflush is controlled by differences in the inlet pressure and the EPC pressure. [A] and [B]: Backflush on: the EPC pressure is higher than the inlet pressure, thus leading to a flow on the guard column in the direction towards the inlet. [C]: Backflush off: the inlet pressure is higher than the EPC pressure resulting in a flow on the guard column towards the MS device. In all cases, and as monitored by the EPC pressure, there is always a flow through the coated capillary column in the direction towards the MS system. [A] Pyrolysis: the sample is flushed for 2 min with synthetic air/helium gas mixture before pyrolysis. The backflush is switched on. [B] Flushing: after pyrolysis the analytes are trapped in the CIS unit and the injection port is flushed with helium for 3 min. The backflush is switched on. [C] Start of the GC run: analytes are released from the CIS unit after further 2 min of flushing. The backflush is switched off. Abbreviations used: CIS = cooled injection system, EPC = electronic pneumatics control, GC = gas chromatograph, MS = mass spectrometer, TDU = thermal desorption unit.

## 1.9 Characterising flavours of cigarettes

Several additives can be used to facilitate the acceptance of the alkaloid bitterness and harshness of the tobacco smoke (Thalhout et al., 2016). No less important, the attractiveness of tobacco products can be enhanced through flavouring agents. Therefore cigarettes and RYO tobacco furnished with characterising flavours became prohibited upon implementation of the TPD. In this Directive characterising flavours are

defined as additives that contribute to a smell or taste other than tobacco in the product, such as fruity (e.g., peach, strawberry), sweet (e.g., vanilla) or menthol-like.

Yet, the exact means by which a particular characterising flavour in cigarettes should be determined is not stipulated by the TPD. Therefore reliable analyses methods need to be developed and adapted to the complex tobacco matrix. Suitable approaches to assess characterising flavours in cigarettes can be derived from both sensory as well as chemical analysis methods.

### 1.9.1 Sensory analyses

If the presence of a flavour needs to be assessed according to the claims outlined in the TPD, a descriptive method should be used for sensory analysis. Such methods need to be conducted by trained expert panels consisting of about 15-50 participants (Thalhout et al., 2016). A commonly used method to evaluate sensory characteristics of products is the Quantitative Descriptive Analysis (QDA) approach. Descriptive analyses are highly time consuming, as panel members need to be trained for several weeks (Krüsemann et al., 2019). In addition, the test procedure itself is also extremely laborious. For instance, in a study conducted by Krüsemann et al. (2019) six smelling sessions with a duration of 1.5 h each were needed to evaluate the profiles of characterising flavours in twenty different tobacco products.

Therefore the evaluation of characteristic flavours in tobacco products should better rely on a combination of both sensory analysis and chemical analysis. The chemical analysis can predict characterising flavourings on the basis of a defined compound profile, and thus should be carried out in the first place.

### 1.9.2 Chemical analyses

For the chemical assessment of characteristic flavours, the complexity of fruity flavours can be challenging. Although some flavours mainly depend on the quality of single compounds (i.e., vanillin, menthol), most fruity flavours are made of a multitude of components acting altogether as a flavouring mixture. Since levels of flavouring compounds in cigarettes are usually less than 0.1%, it may become difficult to distinguish between naturally occurring tobacco flavour compounds and flavours which are added to the product during the manufacture (Purkis et al., 2011).

A widely used method in flavour research is the headspace (HS) GC coupled to mass spectrometry (MS). Headspace analyses are based on the heat dependent distribution of volatile compounds between the sample (solid or liquid) and the gas phase above. Upon heating of the sample, the analytes released to the gas phase can be measured

by using a GC/MS device. A special method of HS analysis is the solid phase microextraction (SPME) (Khataei et al., 2020). This procedure basically consists of two steps, namely the extraction and desorption of the analytes on a sorbent coated fibre. For the HS-SPME the sorbent coated fibre is placed above the sample to adsorb vaporous analytes, which represents an equilibrium-based process. Upon equilibrium between the sample matrix and the extracting phase of the SPME fibre, desorption of the analytes is carried out in the injection port of the GC (Khataei et al., 2020).

If chiral compounds are part of the complex flavour formulations, their enantiomeric ratio also requires attention. Since enantiomeric ratios of naturally derived compounds often differ from synthetic compounds, an enantioselective GC/MS (Es-GC/MS) analysis could be conducted to enable insights on its enantiomeric composition and thus on its origin and authenticity. As a requisite outlined in the TPD, all physicochemical properties of the additives used in tobacco products need to be described, a claim that also includes data on the enantiomeric ratio of chiral compounds.

A unique status among the flavouring compounds used in cigarettes is ascribed to menthol. It is the most commonly used additive in tobacco products and not only applied the case of properly labelled, so-called menthol cigarettes. As outlined in the TPD its use should be regulated not only for its characteristic flavouring properties, but also because this compound most likely contributes to the attractiveness and the addictiveness of tobacco products in serval ways. The way in which menthol contributes to the popularity of certain tobacco products will be discussed in the following below.

## 1.10 Mentholated Cigarettes

Beside vanilla and citrus, menthol is one of the most important flavouring compounds for consumer products (Kamatou et al., 2013). It is widely used as additive in products such as confections, chewing gum, oral care products, etc., as well as medical products.

Menthol is a cyclic monoterpene alcohol with three asymmetric carbons resulting in four enantiomeric pairs, namely menthol, isomenthol, neomenthol, neoisomenthol, and their eight (= $2^3$) stereoisomers (Figure 1-5).

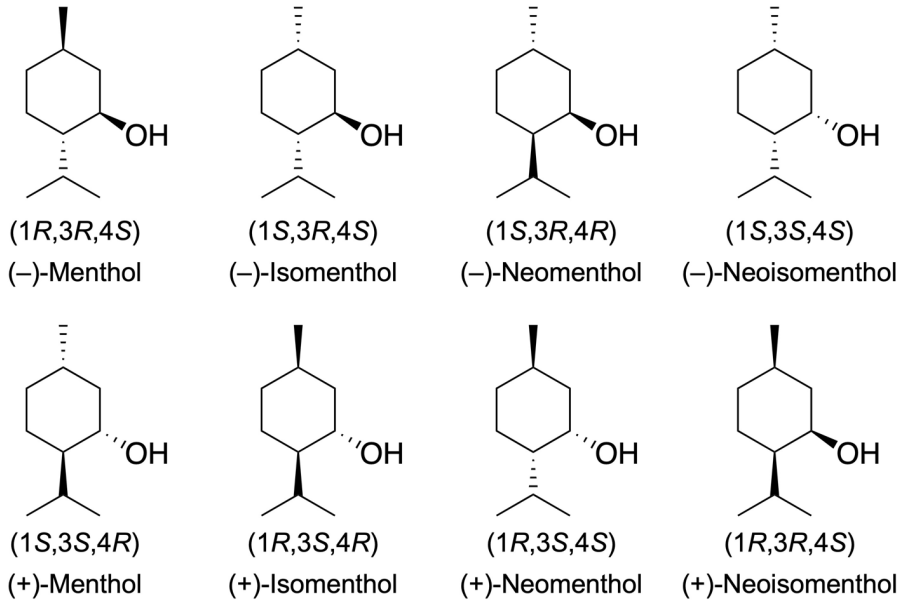


**Figure 1-5: Stereoisomers of menthol (redrawn from Kamatou et al., 2013)**

Naturally derived menthol occurs as (1*R,*3*R,*4*S*)-(–)-menthol in the essential oils of aromatic plants like *Mentha x piperita L.* (Figure 1-6). This isomeric form of menthol has the highest cooling effect and is the preferred ingredient used as additive in the field of consumer products, including tobacco cigarettes. For this purpose, naturally generated menthol is predominant, since the presence of contaminants or residues which might arise during chemical synthesis of menthol, would affect the overall scent of the product (Kamatou et al., 2013). In addition, synthetically produced menthol usually consists of the racemic mixture with only about half of the cooling properties of enantiomerically pure (–)-menthol (Heck, 2010).

**Figure 1-6: *Mentha x piperita L.* (from KÖHLER`S ATLAS der Medizinal-Pflanzen, 1997)**

Mentholated cigarettes were first introduced to the market in the 1920s (Reid, 1993). However, significant increases of its market share failed to appear before the 1950s, a time when health concerns about cigarette smoking already emerged. At that time mentholated cigarettes were merchandised in combination with filters as so-called lower risk cigarettes (Reid, 1993). The rationale behind this was a combination of the menthol-related cooling effect together with low tar contents of the smoke inhaled into the lungs. Even until today, mentholated tobacco products are mistakenly perceived as less harmful (Kopa and Pawliczak, 2020). This assumption relies on the biological effects of menthol as cooling and refreshing agent, which also confers local anaesthetic properties. Based on this, addition of menthol indeed might lead to smoothness and the masking of harshness of the cigarette smoke inhaled by reducing epithelial irritation and suppressing the cough reflex (Kopa and Pawliczak, 2020). Because of these physiological properties of menthol, the inhalation of tobacco smoke into the lungs might be deeper and therefore intensified (Ahijevych and Garrett, 2004, 2010), altogether leading to the enhancement of both the attractiveness and addictiveness of the tobacco product. There is evidence that mentholated cigarettes are preferred by young smokers and might serve as a so-

called gateway product towards nicotine addiction (Giovino et al., 2015). In Europe about 7.4% of cigarette smokers are menthol smokers (Zatonski et al., 2018). In the United States even 39% of the smokers prefer mentholated cigarettes (Jao et al., 2020).

As already mentioned above, menthol can contribute to the addictive potential of cigarettes. Gandhi and co-workers demonstrated that smokers of mentholated cigarettes were less successful in quitting when compared to non-menthol smokers (Gandhi et al., 2009). It has been suggested that this observation might be in part explainable by the interaction of menthol with the nACh receptor. Menthol exposure results in nACh receptor upregulation in the brain under conditions of prolonged and increased bioavailability of nicotine due to the menthol-based inhibition of metabolic degradation of both nicotine as well as cotinine (Kopa and Pawliczak, 2020; Jao et al., 2020).

In addition to these observations related to the addictiveness and attractiveness of tobacco smoke, recent studies suggest that menthol cigarette usage may also be associated with an increased risk for the development of non-cancerous diseases such as cardiovascular diseases and ischaemic insults (“strokes”), as well as the exacerbation of chronic obstructive pulmonary disease (COPD) (Jao et al., 2020).

In Europe, the use of menthol is prohibited as long as its addition to the product creates a characteristic flavour. In Germany the use of menthol as additive is regulated by the TabakerzV. According to this provision menthol is prohibited for cigarettes and RYO tobacco since May 20, 2019, and starting at May 20, 2024, for the remaining tobacco products (TabakerzV, 2016). Also in the United States of America, the FDA recently proposed new tobacco product standards, prohibiting menthol as a characterising flavour in cigarettes in the future (FDA, 2022b). Nevertheless, in the United States and many other countries of the word menthol cigarettes are still available.

In accordance to the TPD, for the regulation of menthol in tobacco products its physiologically active levels in both mentholated as well as non-mentholated cigarettes need to be investigated. These physiological effects of menthol are predominantly related to the activation of the so-called “cold-menthol receptor” (McKemy et al., 2002).

## 1.11 Transient Receptor Potential Cation Channel Subfamily M (Melastatin) Member 8 (TRPM8) receptor

Sensing of pain due to chemical, mechanical or thermal stimuli relies on specialised peripheral sensory neurons (nociceptors) that are present throughout the body. These receptors convert such stimuli into electrical signals, which then are send via the spinal

cord towards higher brain centres (Dubin and Patapoutian, 2010; Geisslinger, 2020). The family of ligand mediated transient receptor potential (TRP) cation channels belong to these nociceptors, and are involved in the sensing of taste, temperature and pain. Today, for the family of TRP cannels seven subfamilies are known to be present in mammals, namely: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPN (Drosophila NOMPC), TRPA (ankyrin), TRPRL (mucolipin), and TRPP (polycystin) (Li, 2017; Teuscher, 2020). The physiological effects of menthol are primarily assigned to the TRPM8 receptor, which is expressed in peripheral sensory neurons to transduce cold stimuli from the environment. This also refers to its expression in the respiratory tract. The TRPM8 is a nonselective cation channel, activated by cold (10-28°C) temperatures, membrane depolarisation, cooling agents (e.g., menthol), changes in osmolality, and changes in the pH-values (Iftinca and Altier, 2020).

# 2 Objective

With the signing of the WHO FCTC all Member States are required to implement reduction strategies in the field of tobacco demand and supply. For the first time a special focus is laid on the regulation of additives used for tobacco products. It has been emphasised that the application of additives shall not contribute to an enhancement of the attractiveness, addictiveness and toxicity of tobacco products (WHO, 2013).

In Europe these objectives have been adopted by the implementation of Directive 2014/40/EU. This Directive requires manufacturers and importers of cigarettes and RYO tobacco to report for each additive whether it

- contributes to or increase the toxicological potential to a significant or measurable degree,
- leads to a characterising flavour of the product,
- facilitates inhalation or nicotine uptake, and
- results in the formation of CMR constituents by itself or increases the CMR properties of the respective tobacco product to a significant or measurable degree.

Still, there is an ongoing debate on how to characterise and measure such properties. Therefore the main subject of this work was to provide reliable methods for the assessment of tobacco additives by pursuing the three central goals outlined in the following:

## 2.1 Smoke simulation studies of tobacco additives

Because tobacco smoke is inherently toxic, the assessment of an additional contribution of tobacco additives to this overall toxicity becomes challenging. High numbers of control and experimental preparations are required to be able to estimate even small changes in compound profiles. Pyrolytic breakdown products are often highly volatile, hence prone to get lost during analyses. Analytical methods for the examination of the toxic potential of tobacco additives should therefore be able to simulate the complex conditions during smoking, enable high sample throughput and should not be prone to compound loss. To this end, a powerful approach of on-line oxidative py-GC/MS has been established to simulate the smoking process and to analyse the chemical species formed.

## 2.2 Chemical analyses of characterising flavours used in cigarettes

Some characterising flavours like vanilla or menthol predominantly rely on single chemical compounds and are thus being rather easy to detect by analytical means. By contrast, most fruity flavours consist of a complex mixture of multiple flavour components. In addition, these compounds often result in a more or less uncharacteristic fruity flavour. To characterise a fruity flavour by analytical means, a combination of different chemical compounds need to be assigned to the flavour. In the present thesis, an HS-SPME-GC/MS method has been developed to propose how analytics might be used to define characterising flavours.

## 2.3 Determine the physiological active levels of menthol

In Europe mentholated cigarettes are prohibited as long as they are responsible for the characteristic mint-like flavour of the product. The issue had to be experimentally addressed whether minor amounts of menthol in non-mentholated cigarettes are sufficient to activate the cold-menthol receptor TRPM8. For that purpose, an analytical method has been established and validated to determine the menthol contents in different tobacco products. Secondary, a cell-based assay was developed to determine the minimum concentrations of menthol required to activate the TRPM8 receptor.

# 3 Results

The experimental work and its results are presented in three separate parts that each have been published in the peer-reviewed literature:

Oxidative and inert pyrolysis on-line coupled to gas chromatography with mass spectrometric detection: On the pyrolysis products of tobacco additives

Study aim: An on-line py-GC/MS method was developed to monitor the occurrence of toxicants formed upon pyrolysis of 19 different tobacco additives. For that purpose, all individual additives were pyrolysed under inert or oxidative conditions at 350, 700 and 1000°C, respectively. Further, the formation of 20 selected polycyclic aromatic hydrocarbons (PAHs) was monitored after pyrolysis of cocoa, that is, a typical tobacco additive, by applying a semi-quantitative approach.

Toward the stereochemical identification of prohibited characterizing flavors in tobacco products: the case of strawberry flavor

Study aim: An HS-SPME-GC/MS technique was developed to characterise different strawberry flavoured tobacco products in terms of their volatile additives. To identify indicative patterns of strawberry flavour compounds in these products, the results were compared to non-flavoured, blend characteristic flavoured and other fruity flavoured cigarettes, as well as fresh and dried strawberries. Among the strawberry flavour compounds detected, selected compounds were further analysed for their stereoisomeric composition by using Es-GC/MS.

Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

Study aim: The minimum concentrations of menthol, linalool, menthone and carvon required to activate the TRPM8 receptor in HEK293 cells were examined using on-line fluorescence microscopy. It was then investigated whether sufficient amounts of these TRPM8 agonists are present in menthol, American blend, additive-free and light cigarettes or dried tobacco leaves, as well as in selected tobacco additives. To determine the contents of menthol and of the other TRPM8 agonists in these products, an HS-SPME-GC/MS method was established and validated.

The central aims, methods and results of this experimental thesis work are being valued and assessed in an overarching and concluding discussion chapter (section #4).

## 3.1 Oxidative and inert pyrolysis on-line coupled to gas chromatography with mass spectrometric detection: On the pyrolysis products of tobacco additives

**Meike Paschke**, Christoph Hutzler, Frank Henkler, and Andreas Luch

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Involvement of the author in this publication: Project planning, project execution, data analysis, writing of the manuscript.

Author contributions as published: MP, CH, FH, and AL contributed to the implementation of this research and designed the study. Experiments and data analyses were performed by MP. MP drafted the manuscript. CH, FH and AL supervised the study, refined and finished the manuscript.

## 3.2 Toward the stereochemical identification of prohibited characterizing flavors in tobacco products: the case of strawberry flavor

**Meike Paschke**, Christoph Hutzler, Frank Henkler, and Andreas Luch

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Involvement of the author in this publication: Project planning, project execution, data analysis, writing of the manuscript.

Author contributions as published: MP, CH, FH, and AL contributed to the implementation of this research and designed the study. Experiments and data analyses were performed by MP. MP drafted the manuscript. CH, FH and AL supervised the study, refined and finished the manuscript.

## 3.3 Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

**Meike Paschke**, Anna Tkachenko, Katja Ackermann, Christoph Hutzler, Frank Henkler, and Andreas Luch

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Author contributions as published: MP, AT, CH, FH, and AL contributed to the implementation of this research and designed the study. Experiments and data analyses were performed by MP, AT and KA. MP drafted the manuscript. AT and FH contributed sections to the draft. CH, FH and AL supervised the study, refined and finished the manuscript.

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# Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

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## HIGHLIGHTS

- Menthol in cigarette smoke activates the cold-receptor TRPM8 and mediates a "cooling effect" that is independent from its mint-like aroma.
- We have assessed the minimum menthol contents in cigarettes required for TRPM8 activation.
- A measurable activation of TRPM8 is expected when the content of menthol exceeds 50 μg per cigarette.

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## ABSTRACT

Activation of the cold-receptor TRPM8 by menthol or other tobacco additives can suppress natural defense reactions such as coughing that usually would become effective as involuntary resistance against the inhalation of fumes. In Europe menthol is only regulated as flavor, but can be used as additive as long as no characteristic mint-like aroma will become noticeable in the end-product tobacco. The question needs to be addressed of whether such comparatively minor contents would be sufficient to trigger a measurable activation of TRPM8.

In this study, we have analyzed both the contents of menthol and other natural TRPM8 agonists in tobacco products and developed a bioassay to determine the minimum concentrations of selected agonists to activate the TRPM8 receptor in cultured cells.

The data confirm menthol as strongest natural agonist investigated. Based on these experiments and previously published data, we have estimated both the minimum menthol concentrations in cigarette smoke and in tobacco that are expected to trigger measurable physiological effects. According to our assessments, TRPM8 activation is likely to occur when cigarettes contain more than 50 micrograms of menthol. Importantly, menthol contents in cigarettes far below the typical levels that require declaration as "mentholated" would be sufficient to activate sensory receptors.



## 1. Introduction

Cigarette mentholation was invented in the 1920s, but the procedure did not gain a higher market share until filtered menthol cigarettes were introduced in the 1950s (Reid, 1993). Even then, marketing strategies combined associations of "cooling effects" with low tar contents (Reid, 1993). Nowadays menthol smokers account for about 30% of cigarette smokers in the USA (TPSAC, 2011). In the United States, mentholated cigarettes are preferred by adolescents (12–17 years) and used by a majority of smokers (56.7%) in this age group, followed by young adults (18–24 years) with a proportion of 45.0%. Notably, a considerable decrease in the ratio of menthol smokers (30.5–34.7%) occurs in the age group of 26 years or older (Giovino et al., 2015). This indicates that beginners start with mentholated products and switch later to alternative products. However, other factors that might affect the preference for menthol cigarettes, as for example as gender, ethnic or cultural background or changing perception of fashionable tobacco products are not yet fully understood. In Europe the proportion of menthol smokers is comparatively low and estimated to about 5%, based on cigarette sales figures (EACH, 2013).

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In light of the summarized data presented in the TPSAC report, mentholated cigarettes seem to attract adolescents and young adults and are thus believed to contribute to smoke initiation (TPSAC, 2011; Rising and Wasson-Blader, 2011). In addition to its contribution as flavoring compound in the aromatization of cigarette smoke, menthol has also physiological properties that might promote the inhalation of tobacco smoke. Sensory effects of menthol result in an increased smoothness and reduced harshness of the smoke; impact effects describe the perception of strength of a cigarette; cooling effects mask the burning/scratching properties of tobacco smoke (Ahijevych and Garrett, 2004, 2010); finally menthol also acts as anaesthetic (Wayne and Connolly, 2004). Importantly, internal documents of the tobacco industry revealed that these effects had been well known (Yerger and McCandless, 2011), and sensory properties were adopted to attract young smokers (Kreslake et al., 2008a, 2008b). Other additives such as menthol esters can be used as substitute for menthol (Bharate and Bharate, 2012) as well. Known physiological effects of menthol in the respiratory tract are bronchodilation, decreased rates of inhalation and prolonged breath holding (Henningfield et al., 2003). Menthol does also suppress tussive irritations in response to fumes (Millqvist et al., 2013) and symptoms of respiratory diseases, such as chronic cough or thick mucus production (Garten and Falkner, 2004). The physiological effects of menthol are predominantly related to the activation of the transient receptor potential melastatin 8 (TRPM8) receptor, which is also named cold-menthol receptor (McKemy et al., 2002). TRPM8 is a member of the transient receptor potential (TRP) cation channels family. TRPM8 is specifically expressed in subpopulations of neurons instrumental in sensing pain and temperature. Sensation of coldness involves various receptors that distinguish innocuous or pleasant stimuli, such as relief from previous heat or irritation, from high-threshold response levels in relation to frost as potentially harmful condition. The menthol receptor TRPM8 is predominantly involved in the transduction of innocuous "cold" stimuli, possibly paralleled by the suppression of heat responses in peripheral neurons (McKemy, 2013). The overall sensation at moderate temperature levels is pleasant and can be mimicked by chemical agonists, regardless of taste or aromatic properties.

Although physiological properties of menthol that can promote the inhalation of harsh tobacco smoke are well defined, there is ongoing debate on its relevance for smoking related health risks (Heck, 2010). Current studies do not support the conclusion that menthol in general would contribute to addictiveness (Fagan et al., 2010) or increases in lung cancer incidence because of much higher exposure levels against nicotine and prototypic carcinogens present in the smoke (Brooks et al., 2003; Carpenter et al., 1999). However, with regard to addictiveness evidence is somehow contradictory (Sidney et al., 1995). Even though menthol might not lead to additional health risks for experienced smokers (Heck, 2009), physiological properties are adequate and indeed sufficient to promote the inhalation of irritating fumes in unexperienced individuals (Rising and Wasson-Blader, 2011), similar as shown in animals (Willis et al., 2011). In support to this assumption, menthol cigarettes had been classified as starter products (Hersey et al., 2006; Kahnert et al., 2012) that are likely to facilitate product's addictiveness in adolescents (TPSAC, 2011). Menthol might increase addictiveness indirectly by two mechanisms. Firstly, impairment of physiological resistance against inhalation of irritating fumes could enable some individuals to start smoking, who otherwise would rather refrain. Secondly, menthol might alter individual smoking habits, leading to a deeper inhalation and thus uptake of higher amounts of nicotine. Again, this might primarily affect beginners who are not yet adapted to a routine inhalation of smoke. In addition, menthol was proposed to delay the metabolism of nicotine (Benowitz et al., 2004).

According to new European regulation, menthol will be restricted as a potential characterizing flavor. This raises the important question whether physiologically active levels of menthol or of other TRPM8 agonists need to be expected in conventional smoking tobacco products that are—explicitly—not sold as "menthol cigarettes" and that do not release the typical mint-like flavor. Menthol was also included in the EU priority list of tobacco additives and is subject of tighter reporting obligations (EU, 2016), partly because of its "cooling" effects". In this manuscript we have assessed whether menthol levels far below the typical contents of declared menthol cigarettes are sufficient to activate TRPM8 *via* inhalation. Our risk assessment is based on the provided experimental data and on previously published studies. We propose that TRPM8 activation can be expected when menthol levels exceed 50 μg per cigarette. These comparatively low amounts are probably adequate to facilitate inhalation, especially during the initiation phase of unexperienced smokers who are not yet adapted to the irritating properties of tobacco smoke. We also provide first data on menthol contents in cigarettes of the German market. In the products analyzed, the proposed limit for physiological effects was only exceeded in cigarettes declared as mentholated.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals, analytical standards and solvents used were of analytical or LC–MS grade. Ethanol (EtOH) (99.9%), poly-L-lysine and sodium chloride (NaCl) were purchased from Merck KGaA (Darmstadt, Germany). ATP (100 mM) was bought from Sigma–Aldrich (Taufkirchen, Germany). Fluo-4 AM (1 mM), HBSS (10x) (Hank's balanced salt solution), lipofectamine 2000 and Opti-MEM were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

### 2.2. Standard substances

All analytical standards were used as racemates (Fig. 1). 7-Hydroxycitronellal (95%), carvone (98%), eucalyptol (99%), geraniol (98%), isopulegol (98%), menthol (99%), menthone (99%) and acetophenone-β,β,β-$d_3$ were purchased from Sigma–Aldrich (Taufkirchen, Germany). Linalool was obtained from Merck KGaA (Darmstadt, Germany) and menthol-$d_4$ from TRC (Ontario, Canada).

### 2.3. Samples and their preparation prior to quantitative analysis

Cigarettes of four major manufacturers were obtained from retailers. From each manufacturer a typical American blend product was used as standard brand. Further, additive-free (*i.e.* non-flavored) and declared menthol variations of these standard brands were used in this study. In addition, modified cigarettes, which are sold under the same brand name but contained 0.6 mg nicotine or less, have been analyzed. The latter products are listed here as "light cigarettes", although this designation was not necessarily used by the manufacturer.

Cocoa and liquorice were also obtained from local retailers. Tobacco plants (*Nicotiana tabacum*) were grown from seeds as reference samples. Dried leafs were further processed without curing, as described for cigarette tobacco. An aliquot of each cigarette (100 mg of tobacco) was exactly weighted in 20 mL headspace vials, followed by addition of 5 mL purified water (saturated with NaCl at 60 °C). To this sample, 5 μL of an internal standard mix was added. In the case of mentholated cigarettes a standard mix containing 200 μg/mL of each menthol-$d_4$ and acetophenone-βββ-$d_3$, dissolved in EtOH, was used, while for all

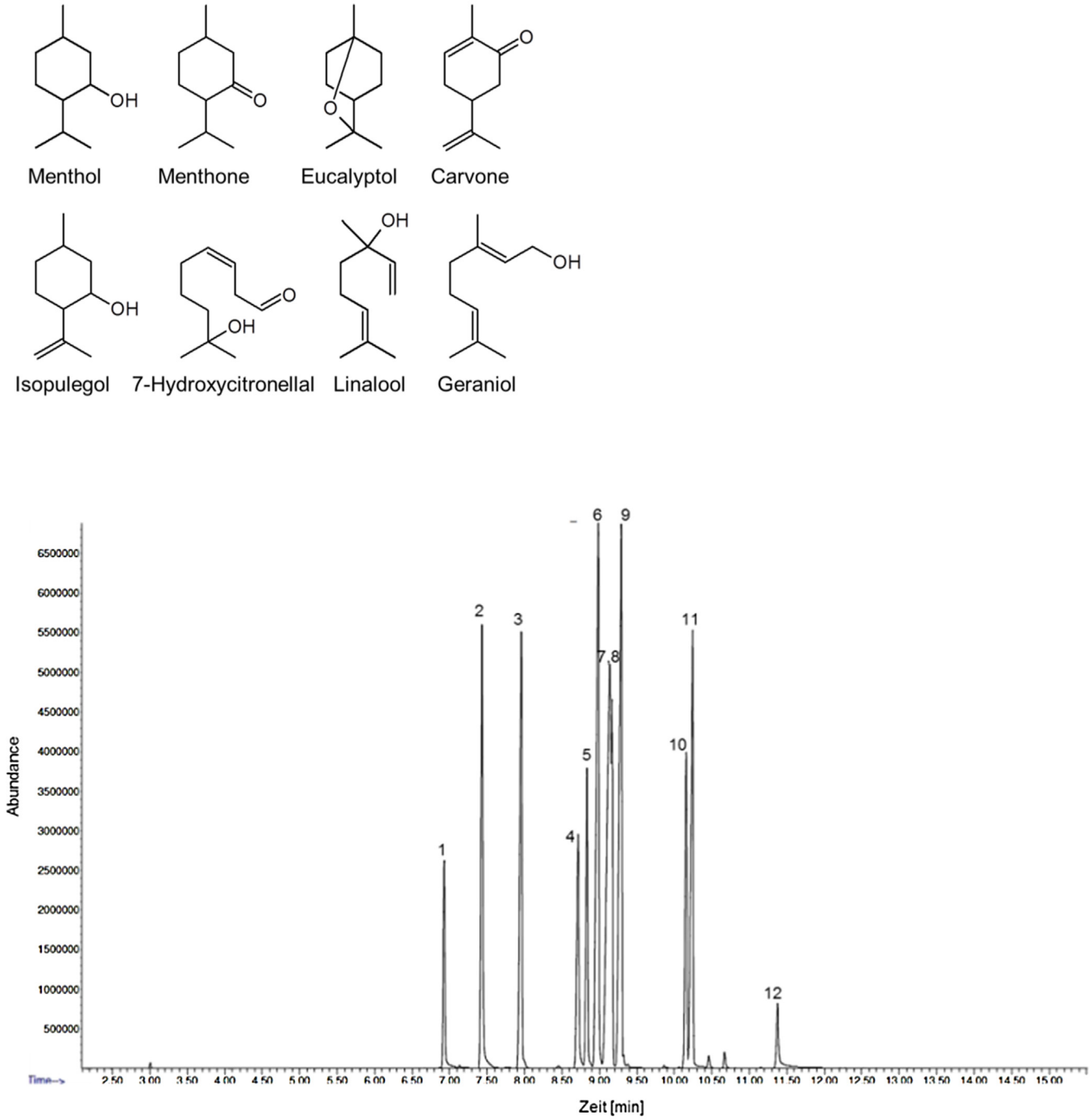


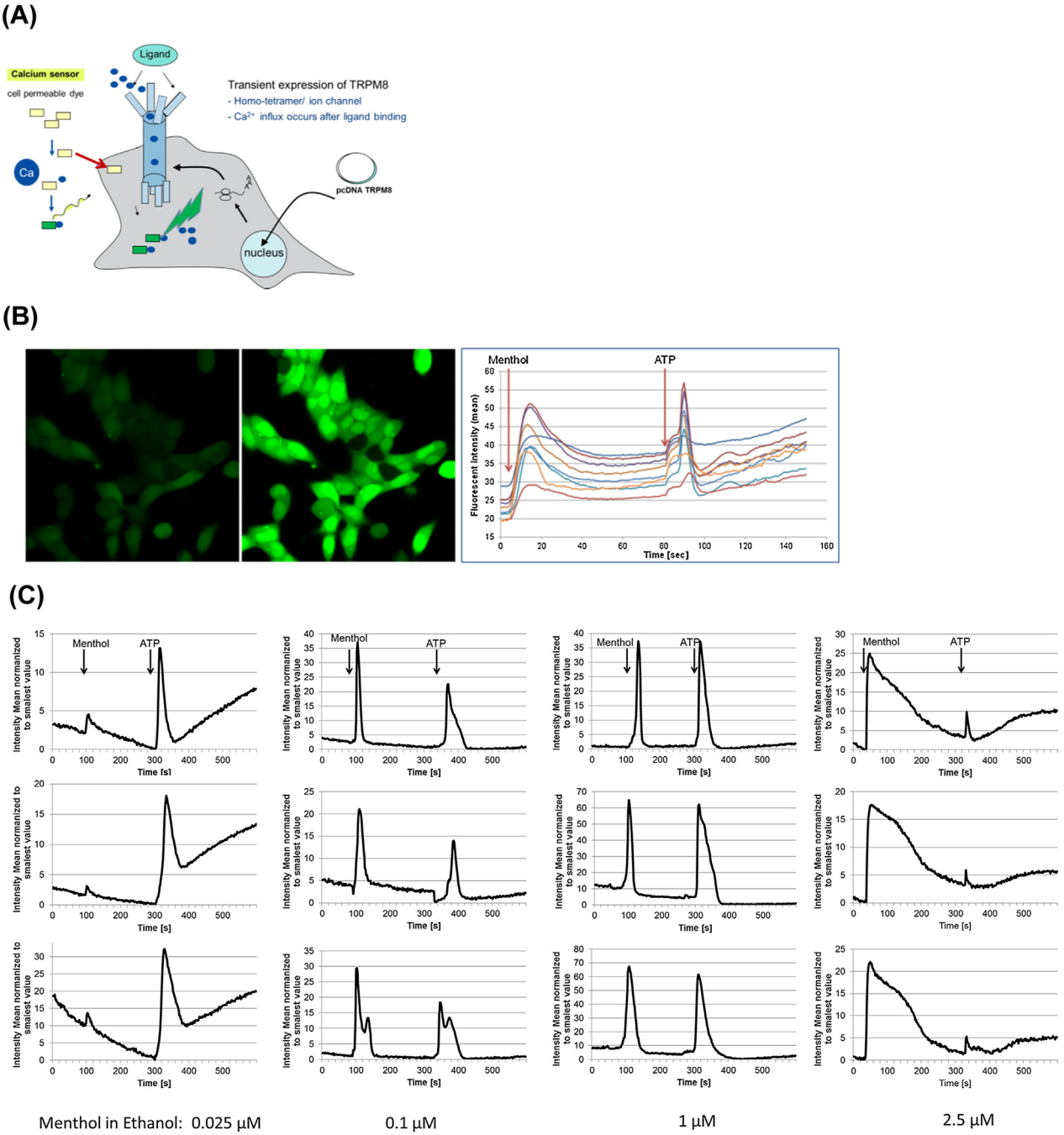

**Fig. 1.** Chemical structures of the TRPM8 agonists analyzed in this study and the corresponding HS-SPME-GC/MS chromatogram of a mixture of these agonists (20 μg/mL of each) along with internal standards (50 μg/mL of each) in SIM mode (see Materials and methods section for details on the HS-SPME–GC/MS procedure). Compounds are labeled as follows: eucalyptol (1); acetophenone-$d_3$ (2); linalool (3); isopulegol (4); menthone (5); neomenthol-$d_4$ (6); menthol-$d_4$ (7); menthol (8); isomenthol-$d_4$ (9); carvone (10); geraniol (11); 7-hydroxycitronellal (12).

other samples this standard mix contained only 50 μg/mL of each ingredient. For quantitative analyses the standard addition method was used and six different calibration points were analyzed for each sample. For mentholated cigarettes 5 μL of standard mix in the range of 200–750 μg/mL EtOH was used for each calibration point. For all other samples a standard mix of 5–150 μg/mL EtOH was added to the sample vials. All samples were analyzed in triplicates and 5 μL of EtOH were added to ensure comparability with the calibration samples. The vials were tightly closed with magnetic silicone/PTFE caps before analysis.

### 2.4. HS-SPME–GC/MS analysis

#### 2.4.1. HS-SPME

For quantification the PDMS/DVB fiber was used as it turned out to be best suited after testing also 4 other fibers with different selectivity (see Supplement, fibers obtained from Supelco (Bellafonte, PA, USA). An MPS2-XL autosampler (Gerstel, Mühlheim, Germany), extended with a SPME fiber holder, was used for automated sampling and injection. The vials were incubated for 5 min at 60 °C. Afterwards, the SPME fiber was exposed to the




| TRPM8 agonist | concentration [µM] | content per cigarette [mg]* |
|---|---|---|
| **Menthol** | **0.1** | **0.00437** |
| Linalool | 100 | 4.32 |
| Menthone | 580 | 25.1 |
| Carvone | 640 | 26.9 |

* Estimate based on a total volume of 280 mL smoke per cigarette (according to DIN ISO 3308)

**Fig. 2.** Panel (**A**): Cartoon of the approach applied in the present study. Panel (**B**): $Ca^{2+}$ imaging of Fluo-4 AM-loaded HEK293 cells. Cells were imaged before (left) and during onset of $Ca^{2+}$ influx (middle). The method was then used for live imaging of several TRPM8 expressing cells that were treated with menthol (25 µM) and ATP (100 µM) at the time points indicated (arrows). Cells were imaged every 2 s and fluorescence was recorded (see Materials and methods section for details). Panel (**C**): Fluorescence signals of TRPM8 expressing HEK293 cells loaded with Fluo-4 AM and treated with menthol at four different concentrations (0.025, 0.1, 1, and 2.5 µM of menthol), followed by 100 µM ATP at the time points indicated (arrows). Cells were continuously imaged every 2 s and fluorescence was quantified in individual cells using the ZEN software provided by Zeiss. Recorded graphs for 3 representative cells are shown for each menthol concentration applied. Non-transfected cells treated with menthol, and TRPM8 expressing cells

headspace for 60 min. Samples were desorbed for 120 s in the cooled injection system (CIS) 4 (Gerstel) at 250 °C, keeping this temperature for 10 min. The CIS was equipped with a bore liner with an inner diameter of 2 mm and operated for mentholated cigarettes with a split ratio of 1:100; all other samples were analyzed in split less mode. Further details on the development of the SPME method are given in the Supplementary Section.

*2.4.2. GC/MS*

A GC 6890A coupled to the MSD 5975C (Agilent, Waldbronn, Germany) equipped with an HP–5 ms column (30 m × 0.25 mm × 0.25 μm, J&W Scientific, Folsom, CA, USA) was used. Helium gas (purity 99.999%) from Air Liquide (Düsseldorf, Germany) was used as carrier gas at a constant flow of 1 mL/min. The oven temperature of the GC was programmed to start at 50 °C, held for 1 min, and then increased with a rate of 10 °C/min to 180 °C, hold for 1 min, followed by 20 °C/min to reach the final temperature of 320 °C, then held for further 1.5 min. The temperatures of the transfer line, quadrupole and the ion source were 295 °C, 150 °C and 230 °C, respectively. The MS was operated in the combined selective ion monitoring (SIM) scan mode. The mass range was scanned in full scan mode in the mass to charge (*m*/*z*) range from 29 to 300 Da. For data acquisition in SIM mode the monitored ions were divided into four groups according to the retention times of the corresponding analytes, with a dwell time of 10 ms for each, using the quantifier and qualifier ions. All analytes under consideration were identified by comparison of their mass spectra and retention times to those of authentic standards (Fig. 1). Data on retention times, quantifiers and qualifiers, limits of detection (LODs) and limits of quantification (LOQs) as well as the description of method validation are given in the Supplementary section (Tables S1–S3) also the method development. Data were evaluated using MSD ChemStation software, Version E.01.00.237 (Agilent, Waldbronn, Germany).

*2.5. TRPM8 cloning*

The human TRPM8 cDNA was optimized for expression in human cells and synthesized and sequenced by Eurofins Genomics (Ebersberg, Germany). The synthesized TRPM8 fragment was subcloned into the pcDNA3 vector (Invitrogen) using the *Eco*RV/*Not*I sites thereby generating the pcDNA3-TRPM8 plasmid. Sequences of the modified cDNA and the translated protein (http://web.expasy.org) are given in Supplementary Fig. S1. Sequence alignment, using the protein Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins), confirmed 100% conformity of the encoded protein with human TRPM8 (NCBI reference sequence NP_076985.4). CellLight nucleus-CFP BacMam 2.0 was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

*2.6. Cell culture media*

Dulbecco's phosphate-buffered saline (DPBS), Dulbecco's Modified Eagle's Medium (DMEM) culture medium, containing 10% fetal calf serum (v/v), L-glutamine (2 mM), and penicillin/streptomycin (100 U/mL).

*2.7. Calcium imaging*

HEK293 cells were seeded at a density of 150,000 cells/dish onto glass bottom dishes coated with poly-L-lysine (dish size of 35 mm, well size of 20 mm; IBL, Gerasdorf, Austria). Cells were grown to confluency in DMEM with 10% FCS at 37 °C and 5% $CO_2$. Confluent cell cultures were then co-transfected with human TRPM8 and CFP-Nuc as nuclear marker. Expression of TRPM8 was confirmed in control experiments by immunofluorescence, using a rabbit polyclonal antiserum (HPA024117, Sigma) according to the instructions of the manufacturer (Supplementary Fig. S1).

Twenty-four hours after transfection, HEK cells transiently expressing the TRPM8 receptor were treated with the cell permeable calcium indicator Fluo-4 AM. Binding to calcium ions converts this compound into a fluorescent dye ($\lambda_{ex}$ 488 nm), thus allowing to monitor calcium influx into cells or the release from endoplasmic reticulum or Golgi apparatus. Prior to treatment, cells were first washed three times with HBSS and then 0.5 mL Fluo-4 AM (2 μM in HBSS) was loaded onto the cells. After 30 min of incubation at room temperature the cells were washed again three times with HBSS. Then 1 mL HBSS was added and cells were incubated for further 30 min at room temperature before being analyzed under the fluorescence microscope. A time series of 600 images during 300 s was recorded. For $Ca^{2+}$ imaging, the agonists were dissolved in EtOH and 1 mL was added onto the transfected and Fluo-4 AM-loaded HEK293 cells after a few seconds (Fig. 2). Approximately 150 s later 0.5 mL ATP solution (100 μM in EtOH) was added to the cells as control. ATP non-specifically opens ion channels and thus provides a reproducible fluorescent signal. The fluorescent signals of at least 10 cells were evaluated. Transfection efficiencies of >80% were confirmed in all experiments, using CFP-Nuc as marker (Thermo Fisher Scientific, Waltham, MA, USA).

## 3. Results

*3.1. Contents of TRPM8 agonists in cigarettes, tobacco leafs and tobacco additives*

Flavoring compounds such as menthol can trigger the activation of the TRPM8 nociceptor for cold pain. Besides menthol, other monoterpenes are described in the literature to activate TRPM8 as well (Behrendt et al., 2004). Behrendt and coworkers demonstrated TRPM8 activation by geraniol, linalool, eucalyptol, isopulegol and 7-hydroxycitronellal in mice. Menthone and carvone are further known agonists (Bharate and Bharate, 2012). In the present study the contents of these selected eight TRPM8 agonists (Fig. 1) were analyzed in four different cigarette brands. Of each brand different types of cigarettes were investigated: American blend cigarettes, additive-free cigarettes, nicotine reduced "light" cigarettes and mentholated cigarettes. The results are compiled in detail in the Supplementary section (Tables S4–S7) and summarized in Table 1.

In mentholated cigarettes we could detect eucalyptol, linalool, isopulegol, menthone, menthol and carvone, but no 7-hydroxycitronellal or geraniol (Table 1). Except for menthol, which was quantified in the milligram range (0.52–4.19 mg/cigarette), all analytes were detected at microgram levels in the cigarette brands investigated. Our data are comparable to the results published by Ai and coworkers in 2016, who analyzed mentholated and non-mentholated cigarettes of the US market (Ai et al., 2016). In mentholated cigarettes they reported an average menthol level of 4.75 mg per cigarette.

In American blend cigarettes only the four analytes linalool, menthone, menthol and carvone could be detected (Table 1). Additive-free cigarettes also contained eucalyptol and geraniol. The detected levels of linalool, menthone, menthol and carvone were about the same in American blend and additive-free

treated with ethanol (solvent) served as controls. The table beneath summarizes minimum levels for TRPM8 activation as determined for menthol, linalool, menthone and carvone using this cell culture assay, and the estimated agonist contents per cigarette required to trigger TRPM8 activation *in vivo* (according to the parameters laid down in the ISO smoking regime, DIN ISO 3308; cf. Discussion section).

**Table 1**
Contents of TRPM8 agonists in cigarettes (n = 4), tobacco leaf and selected additives.

| Compound | American blend cigarettes ng/cig | Additive-free cigarettes ng/cig | Light cigarettes ng/cig | Menthol cigarettes μg/cig | Tobacco leaf μg/g[a] | Cocoa μg/g[a] | Licorice μg/g[a] |
|---|---|---|---|---|---|---|---|
| Eucalyptol | n.d. | n.d.–40.5 | n.d. | 0.15–10.6 | n.d. | n.d. | 0.66 (9) |
| Linalool | 98.2–143 | 75.8–229 | 138–261 | 0.67–4.05 | 0.31 (220) | 0.31 (4.3) | 0.88 (12) |
| Isopulegol | n.d. | n.d. | n.d. | 0.73–6.00 | n.d. | 0.47 (6.5) | n.d. |
| Menthone | 35.9–37.9 | 22.6–103 | 53.3–153 | 1.38–37.8 | n.d. | n.d. | 4.21 (59) |
| **Menthol** | **58.2–445** | **23.4–256** | **121–3640** | **516–4190** | **0.30 (210)** | **0.20 (3.0)** | **0.65 (9.0)** |
| Carvone | 58.9–88.3 | 36.2–76.9 | 43.2–132 | 0.37–270 | 0.28 (200) | n.d. | 1.41 (20) |
| Geraniol | n.d. | 67.7–125 | 87.4–206 | n.d. | n.d. | n.d. | n.d. |
| 7-Hydroxy-citronellal | n.d. | n.d. | 50.4–190 | n.d. | n.d. | n.d. | n.d. |

n.d.: not detected; cig: cigarette; data for menthol are marked in bold.
[a] For tobacco leaf, cocoa and licorice estimations on the contents per cigarette (expressed as ng/cigarette) are provided in brackets. Estimations are based on 700 mg tobacco per cigarette and proportions of 2% for each cocoa and licorice).

cigarettes, a result that indicates that no TRPM8 agonists were voluntarily added to the respective American blend cigarette brands by the manufacturers. However, contents of menthol were found at significantly higher levels in light cigarettes (Table 1). Wayne and Connolly (2004) already described that the amounts of menthol were generally higher in light cigarettes when compared to regular ones. Accordingly, in our study menthol has been quantified in American blend cigarettes and additive-free cigarettes in the range of 58–445 and 23–256 ng/cigarette, respectively. By contrast, in light cigarettes up to 3.6 micrograms menthol were found in the brands investigated. Based on these data, it seems likely that menthol and other TRPM8 agonists have been applied with the intention to enhance the flavor of light cigarettes. Besides linalool, menthone, menthol and carvone also geraniol and 7-hydroxycitronellal were detectable in light cigarettes (Table 1).

We have further analyzed self-grown smoking tobacco to get some idea on endogenous levels of menthol possibly being present in the leaves. In the dried leaves we could detect linalool, menthol and carvone, all of which at about 0.3 μg/g tobacco (Table 1). Based on an average tobacco content of 700 mg/cigarette this would result in 210 ng/cigarette of each of the detected compounds. These levels would be comparable to the concentrations detected in the American blend, additive-free and—except for menthol—also the light cigarettes. Besides endogenous levels in tobacco, natural TRPM8 agonists can also emerge as ingredients of natural additives, including cocoa and licorice. We therefore analyzed the levels of the eight selected TRPM8 agonists in these both kinds of additives as well (Table 1). In cocoa we found linalool, isopulegol and menthol, whereas eucalyptol, linalool, menthone, menthol and carvone were detectable in licorice. Our results show that TRPM8 agonists, such as those monoterpenes analyzed, are present in untreated tobacco leaves, but can also additionally be introduced into cigarettes through additives. However, the overall effect is expected to be rather limited, because these additives do only account for a comparatively low percentage of the total weight.

### 3.2. Assessment of minimum menthol levels required for activation of TRPM8

Detectable levels of TRPM8 agonists in tobacco products are not necessarily related to receptor activation during smoking. It is therefore important to estimate concentrations and levels of particular agonists that are required for on-target activation of TRPM8. Although TRPM8 activation by various agonists had been analyzed before (Behrendt et al., 2004; Bödding et al., 2007), no minimum levels have yet been determined. In this study, we have transiently expressed TRPM8 in HEK293 cells. Although TRPM8 could not be directly visualized in living cells, successful transfections were confirmed by parallel expression of a fluorescent nuclear marker protein. Cell populations were then labelled with the calcium sensor Fluo-4 AM as described in the method section and monitored by online fluorescence microscopy (Fig. 2A). Binding of calcium ions to the sensor led to the emission of green light when the samples get excited at 488 nm. These signals were recorded every 2 s as an indicator for calcium influx. With this approach, a mixed population was analyzed at the level of individual cells (represented as graphs in Fig. 2B). Application of 25 μM menthol led to a strong, but transient influx of calcium in the transfected cells, indicating activation of TRPM8. Non-specific activation of receptors and subsequent calcium influx was then triggered by treatment with ATP as control (Fig. 2B). Comparable responses were observed in the analyzed cell populations, despite some variations in the basal calcium levels between individual cells.

Next, we analyzed TRPM8 activation in cells by gradually lowering the menthol concentrations from 2.5 μM to 0.025 μM (Fig. 2C). Notably, there was no clear dose response in relation to the initially induced calcium influx. However, at higher concentrations (2.5 μM) a prolonged influx of calcium has been observed, while recuperation of basal levels was delayed. In contrast, only a marginal $Ca^{2+}$ increase was observed after application of 0.025 μM

**Table 2**
Reported activation of cold-receptors by defined menthol concentrations/levels.

| Study/publication | Experimental details | Effective menthol concentration/content |
|---|---|---|
| Sant' Ambrogio et al. (1991) | Activation of laryngeal cold-receptors in eleven anesthetized dogs after nasal inhalation of L-menthol. Activation of cold-receptors was directly analyzed at internal branches of laryngeal nerves. | 140–390 ng/mL = 0.9–2.8 μM |
| Behrendt et al. (2004) | Transient expression of murine TRPM8 in HEK293 cells. Determination of $Ca^{2+}$ influx using the fluorometric imaging plate reader (FLIPR) assay. | 4.1 ± 1.3 μM ($EC_{50}$, (−)-isomer)<br>14.4 ± 1.3 μM ($EC_{50}$, (+)-isomer) |
| Bödding et al. (2007) | Stable expression of human TRPM8 in HEK293 cells. Determination of $Ca^{2+}$ influx using microfluorimetry. | 10.4 μM ($EC_{50}$) |
| Willis et al. (2011) | Mice were challenged with smoke irritants (acrolein). The irritation response was completely blocked by co-inhalation of menthol. | strong inhibition at 16 ppm (0.65 μM); 2 ppm had no effect |

menthol. Our data suggest 0.1 μM menthol as minimum concentration to activate TRPM8 at the molecular level.

## 4. Discussion

As expected, our estimated minimum menthol concentration required for TRPM8 activation is lower than the previously determined $EC_{50}$ values *in vitro* (Behrendt et al., 2004; Bödding et al., 2007) (Table 2). However, this estimated minimum level is in about the same range as menthol concentrations that had previously been reported to trigger a detectable activation of cold-receptors *in vivo* (Willis et al., 2011; Sant' Ambrogio et al., 1991) (Table 2).

Willis et al. (2011) demonstrated that inhalation of 16 ppm menthol did completely block the irritation response toward acrolein, whereas 2 ppm menthol showed no effect. This corresponds to an active menthol concentration of approximately 0.65 μM, which is about 6-fold higher than our estimated minimum level. We conclude that the threshold level of menthol in tobacco smoke sufficient to trigger a relevant and detectable TRPM8 activation lies somewhere between 0.1 μM and 0.65 μM. Experiments by Sant' Ambrogio et al. (1991) confirmed a similar value, demonstrating that 0.9 μM menthol (140 ng/mL) was sufficient to activate cold-sensing laryngeal receptors/neurons in new born dogs. To assess physiological effects including putative risks of menthol in tobacco products, we postulate a minimum menthol concentration required to trigger relevant *in vivo* effects between 0.3–0.4 μM. In addition, we have also determined the minimum levels of linalool, menthone and carvone for TRPM8 activation (Fig. 2). The numbers obtained confirm that these compounds are less potent than menthol (Behrendt et al., 2004).

Although physiological effects of menthol on cold-receptors are well recognized, the regulation as tobacco additive is still focused on its mint-like flavor. Importantly, activation of TRPM8 needs to be regarded as relevant mechanism to suppress irritating properties of tobacco smoke, and thus as some sort of counteraction of the natural resistance against the inhalation of fumes. In principle, detectable activation of TRPM8 constitutes an additional risk for smokers, despite the likely differential relevance for beginners and experienced smokers. Several investigations and studies provide evidence for a supporting role of menthol during smoking initiation, amongst these—most importantly—the ascertainment of the high prevalence of mentholated products among youths (TPSAC, 2011). There is further evidence indicating that people who start smoking by using mentholated cigarettes are more likely to progress toward regular smoking habits and nicotine dependence (Nonnemaker et al., 2013). This assumption is also supported by higher scores of individuals of this population when nicotine-dependence scales are being applied (Hersey et al., 2006; Fagan et al., 2015). According to previous studies, menthol does not affect long-term health (Brooks et al., 2003; Carpenter et al., 1999), a finding especially relevant in the case of lung cancer disease which mainly affects smokers who had been adapted (and possibly addicted) for a comparatively long time. After adaption, the physiological properties of menthol and other TRPM8 agonists therefore seem less relevant to maintain smoking habits.

In consideration of the data presented, we propose that tobacco products and additives should be assessed in terms of both flavor and putative physiological effects. Based on the parameters of the ISO smoking regime (DIN ISO 3308, see: http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=60404), the proposed minimum menthol concentration of 0.1 μM–0.65 μM would relate to 4.37 μg–28.4 μg per 280 mL (that is, the smoking volume of one cigarette: 8 puffs, 35 mL each). According to the European Tobacco Product Directive, DIN ISO 3308 is used to analyze emissions; however machine smoking regimes can only provide a rough simulation of human smoking behavior. Menthol as flavoring compound is highly volatile; however, little is known on the transition of low-level menthol from dry tobacco into tobacco smoke. For a rough estimate, we considered the analysis of Gordon et al. (2011) who demonstrated a significant transfer of menthol into tobacco smoke, using 0.1% mentholated cigarettes. Notably, menthol was associated with the total particulate matter, while transfer in the gas phase was not determined. This suggests that elevated concentrations might occur in the upper airway by inhalation of particles that carry menthol. Assuming an approximate transfer of 30%, which was also confirmed by earlier studies (Bozinski et al., 1972), minimal menthol contents of about 15–95 μg per cigarette would be expected to trigger a noticeable activation of TRPM8 in the respiratory tract of individuals while smoking. Although data gaps still need to be filled, our calculations allow for some further conclusions. Firstly, even menthol levels far below the milligram scale, the latter well known to be regularly applied in mentholated cigarettes (Ai et al., 2016) (cf. Table 1), presumably will be well sufficient to trigger TRPM8-mediated effects during inhalation. According to our model, TRPM8 activation is likely to occur when menthol contents exceed 50 μg per cigarette. Although the threshold for partial TRPM8 activation might be lower, TRPM8 activation probably can be excluded when menthol contents fall below 4 μg per cigarette. Secondly, endogenous menthol levels are unlikely to trigger TRPM8 activation. Again only limited data are available. Our investigation of self-grown tobacco plants reveals an endogenous content of approximately 0.3 μg menthol per gram tobacco leaf (Table 1), or 0.21 μg per cigarette. Even in a worst case estimate (onset of relevant TRPM8 activation at 4.37 μg per cigarette), endogenous contents thus would still be 20-fold below physiologically relevant levels. Nevertheless, endogenous menthol contents of industrially processed tobacco should be monitored by product surveillance, keeping in mind that newly bred or genetically modified tobacco might end up with much higher endogenous menthol levels as our self-grown plants. Thirdly, other natural TRPM8 agonists, especially linalool, menthone and carvone require 1000–6500-fold higher levels to trigger similar effects on the cold-receptor as menthol (Fig. 2). Nevertheless it seems feasible to achieve TRPM8 activation if comparatively high levels were to be used as additives. On the other side, some synthetic compounds, especially the TRPM8 super-agonist icilin [*i.e.*, 1-(2-hydroxyphenyl)-4-(3-nitrophenyl)-3,6-dihydropyrimidin-2-one], may mediate similar or even stronger effects compared to menthol (Behrendt et al., 2004). There is as yet no hint that such highly potent TRPM8 agonists already advanced into practical application though.

## 5. Conclusions

Although we did not find menthol levels sufficient to activate TRPM8 in the non-mentholated products analyzed here (highest levels of about 3.6 μg/cigarette were found in light cigarettes, brand A; see Table S7), others did (Ai et al., 2016; Richter et al., 2016). Hence some concern exists that products might be adjusted to avoid a mint-like characterizing flavor on the one hand, but remain to be capable of triggering a cooling effect within the upper respiratory tract on the other. Conversely, all of the mentholated cigarette brands investigated (see Table S4), revealed menthol levels well beyond the level of 50 μg per cigarette and are thus likely capable of inducing TRPM8 activation *in vivo*. In principle, agonists of the TRPM8 receptor are suited to contribute to the enhancement of tobacco smoke inhalation *via* defined and detectable physiological effects in the respiratory tract. This premise remains valid, although the relevance of TRPM8 activation to enhance inhalation seems less apparent for experienced

smokers after adaption. Still, the tobacco industry might be tempted to continue efforts to utilize synthetic compounds as alternative cooling agents, being much more potent than the natural menthol-like flavorings that were in the focus so far (Bharate and Bharate, 2012). European regulators should consider further restrictions for menthol and synthetic TRPM8 agonists, based on its innate physiological properties at the TRPM8 receptor.

## Conflict of interest

None

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2017.02.020.

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**SUPPLEMENTARY INFORMATION:**

# Activation of the cold-receptor TRPM8 by low levels of menthol in tobacco products

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(This file contains 5 supplemental figures and 7 supplemental tables)

## Table of Contents:

**Fig. S1:** Details and verification of the TRPM8 expression vector

**(A)** Sequence of the synthesized TRPM8 gene: The coding sequence is shown in capital letters, both start and stop codons are underlined. The sequence was cloned into pcDNA3 (Invitrogen) using the *Eco*RV / *Not*I restriction sites.

gat atc ctc gag aag gcc acc ATG AGC TTC AGA GCT GCC CGA CTC AGC ATG AGG AAT CGG CGC AAC GAT ACT CTG GAT TCT ACC AGA ACC TTG TAC TCC TCC GCT TCA AGG TCT ACT GAT CTG AGC TAT AGC GAA AGC GAT CTG GTG AAC TTC ATT CAG GCC AAT TTC AAG AAA AGG GAG TGT GTG TTC TTC ACT AAA GAC AGT AAA GCC ACA GAG AAC GTG TGC AAG TGT GGG TAC GCC CAA AGT CAA CAC ATG GAA GGA ACC CAG ATT AAT CAG TCC GAG AAG TGG AAC TAC AAG AAG CAC ACT AAG GAA TTC CCT ACC GAC GCC TTT GGG GAC ATT CAG TTT GAA ACT CTG GGC AAG AAG GGA AAG TAC ATT CGG CTC TCT TGC GAC ACC GAT GCC GAG ATT TTG TAT GAG CTG CTT ACG CAA CAT TGG CAT CTC AAG ACC CCT AAC CTG GTG ATC AGT GTT ACT GGC GGG GCC AAG AAC TTT GCC CTG AAG CCA CGG ATG CGT AAA ATC TTC TCT CGC CTC ATT TAC ATT GCG CAG TCA AAG GGC GCT TGG ATT TTG ACA GGC GGC ACA CAC TAC GGT CTG ATG AAG TAC ATT GGA GAG GTG GTT CGC GAC AAT ACC ATT AGC CGC AGT AGT GAG GAG AAC ATC GTC GCC ATC GGT ATT GCA GCT TGG GGC ATG GTT TCT AAC CGT GAT ACC TTG ATA AGG AAC TGC GAC GCC GAA GGG TAT TTT CTT GCA CAG TAT TTG ATG GAC GAC TTT ACC CGA GAT CCC CTC TAT ATC CTG GAT AAC AAC CAC ACA CAT TTG CTC CTT GTG GAT AAC GGT TGT CAC GGG CAT CCT ACT GTC GAA GCG AAG CTG CGC AAC CAG CTT GAG AAA TAC ATT TCC GAG AGA ACG ATC CAG GAC TCA AAT TAC GGT GGC AAG ATC CCA ATC GTG TGT TTT GCA CAA GGA GGA GGG AAA GAG ACT CTG AAA GCT ATA AAT ACC TCA ATC AAA AAC AAG ATC CCA TGC GTA GTT GTA GAA GGC TCT GGA CAG ATT GCG GAC GTG ATC GCA TCA TTG GTA GAA GTA GAG GAT GCT CTG ACG TCT AGC GCA GTG AAA GAG AAG CTT GTG CGC TTT CTC CCC AGG ACA GTG AGT AGA CTC CCC GAG GAG GAA ACT GAG AGC TGG ATT AAG TGG CTG AAA GAA ATC CTG GAA TGC TCA CAT CTG CTG ACG GTT ATT AAG ATG GAA GAG GCT GGC GAC GAG ATC GTG TCA AAC GCT ATA AGC TAT GCT CTG TAC AAA GCC TTC AGC ACA TCC GAA CAG GAC AAG GAC AAC TGG AAT GGG CAG TTG AAA CTC CTC CTG GAA TGG AAT CAG CTC GAT CTG GCC AAT GAT GAG ATC TTT ACC AAT GAC AGG AGG TGG GAA TCA GCG GAT CTG CAG GAG GTG ATG TTT ACC GCC CTC ATC AAA GAC AGA CCC AAG TTC GTG AGG CTG TTC TTG GAG AAT GGG CTG AAT CTC AGA AAA TTC CTG ACA CAT GAT GTG CTG ACA GAG CTG TTC AGC AAC CAC TTT AGC ACA CTG GTT TAT CGG AAT CTG CAG ATC GCA AAG AAC TCC TAT AAT GAT GCC TTG CTT ACA TTC GTG TGG AAA CTG GTG GCC AAT TTT AGA CGG GGA TTT CGG AAA GAG GAT AGA AAT GGC CGG GAT GAA ATG GAC ATT GAG TTG CAT GAC GTG AGT CCC ATC ACG AGA CAC CCA CTC CAG GCT CTG TTC ATT TGG GCT ATT CTG CAG AAC AAG AAA GAA CTG TCC AAA GTC ATT TGG GAA CAG ACA AGA GGA TGT ACC CTC GCT GCC CTG GGT GCC AGT AAA CTT CTT AAG ACA CTG GCG AAA GTC AAG AAT GAC ATA AAT GCT GCC GGG GAG TCC GAG GAA CTT GCT AAC GAG TAC GAA ACA AGG GCA GTG GAG CTG TTC ACC GAA TGC TAC TCT AGC GAC GAG GAC TTG GCT GAA CAA CTG CTG GTG TAT AGC TGC GAA GCA TGG GGC GGG TCT AAT TGC CTG GAG CTG GCT GTT GAA GCA ACC GAT CAG CAC TTT ATC GCA CAA CCA GGA GTC CAG AAT TTC CTT TCC AAA CAA TGG TAT GGC GAA ATA TCT CGG GAT ACT AAG AAT TGG AAA ATT ATT TTG TGC CTG TTC ATT ATT CCC CTG GTT GGA TGT GGC TTT GTG AGC TTC CGC AAG AAA CCC GTG GAC AAA CAC AAG AAG CTC CTG TGG TAC TAC GTG GCT TTC TTT ACG TCA CCC TTT GTC GTG TTT AGT TGG AAC GTA GTA TTC TAC ATT GCC TTC CTC CTG CTG TTT GCC TAT GTG CTC CTG ATG GAC TTC CAT TCT GTG CCT CAC CCT CCA GAG CTC GTG CTG TAC AGC CTG GTG TTT GTT CTT TTC TGC GAT GAG GTT CGC CAA TGG TAT GTC AAT GGG GTC AAT TAC TTT ACT GAC CTC TGG AAT GTG ATG GAC ACC CTG GGG TTG TTT TAC TTC ATA GCG GGT ATC GTA TTC CGA CTG CAT TCT AGC AAC AAA TCC AGC CTG TAT TCC GGC CGG GTC ATA TTC TGT CTG GAC TAT ATC ATC TTC ACA CTG AGA CTT ATC CAC ATA TTC ACA GTG TCA CGT AAT CTC GGC CCC AAG ATC ATC ATG CTG CAG AGG ATG CTG ATA GAT GTT TTC TTC TTC TTG TTT CTG TTC GCA GTC TGG ATG GTT GCC TTC GGT GTC GCG CGG CAG GGA ATA CTT CGA CAG AAT GAG CAA CGT TGG CGA TGG ATC TTC AGA TCT GTG ATC TAC GAA CCA TAC CTT GCC ATG TTT GGT CAG GTA CCT TCC GAC GTA GAT GGC ACT ACC TAT GAT TTT GCA CAC TGT ACC TTT ACC GGA AAT GAA AGC AAA CCG CTT TGT GTT GAG CTG GAT GAA CAC AAC CTC CCT CGG TTT CCG GAA TGG ATC ACC ATC CCA CTG GTC TGT ATA TAT ATG CTG TCC ACA AAC ATC CTG CTG GTG AAC TTG CTC GTC GCA ATG TTC GGA TAT ACG GTG GGC ACA GTG CAG GAG AAC AAC GAT CAG GTC TGG AAA TTT CAG CGC TAT TTT CTG GTG CAA GAG TAC TGT TCC AGG CTG AAT ATC CCT TTC CCG TTT ATC GTC TTT GCC TAC TTT TAC ATG GTC GTT AAG AAA TGC TTC AAA TGC TGC TGC AAG GAA AAG AAC ATG GAA AGC TCC GTG TGC TGT TTC AAG AAC GAG GAT AAT GAG ACT CTG GCC TGG GAG GGC GTC ATG AAG GAG AAT TAT CTG GTC AAA ATC AAC ACC AAG GCA AAC GAC ACT AGT GAG GAG ATG AGG CAT CGA TTT CGC CAG CTG GAC ACT AAA CTT AAC GAC CTG AAG GGG CTC CTG AAA GAG ATA GCC AAC AAG ATT AAG TGA ccg cgg ctc gag gcg gcc gc

**(B)** Translation and confirmation: The sequence was translated using the ExPASy-Translate tool (http://web.expasy.org/translate/). The protein sequence was then analyzed using BLAST (Basic Local Aligment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) to confirm a 100% homology with human TRPM8.

Range 1: 1 to 1104 GenPeptGraphics Next Match Previous Match

Alignment statistics for match #1

| Score | Expect | Method | Identities | Positives | Gaps |
|---|---|---|---|---|---|
| 2295 bits(5947) | 0.0 | Compositional matrix adjust. | 1104/1104(100%) | 1104/1104(100%) | 0/1104(0%) |

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synthTRPM8  1     MSFRAARLSMRNRRNDTLDSTRTLYSSASRSTDLSYSESDLVNFIQANFKKRECVFFTKD  60
                  MSFRAARLSMRNRRNDTLDSTRTLYSSASRSTDLSYSESDLVNFIQANFKKRECVFFTKD
humanTRPM8  1     MSFRAARLSMRNRRNDTLDSTRTLYSSASRSTDLSYSESDLVNFIQANFKKRECVFFTKD  60

synthTRPM8  61    SKATENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRL  120
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humanTRPM8  61    SKATENVCKCGYAQSQHMEGTQINQSEKWNYKKHTKEFPTDAFGDIQFETLGKKGKYIRL  120

synthTRPM8  121   SCDTDAEILYELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWIL  180
                  SCDTDAEILYELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWIL
humanTRPM8  121   SCDTDAEILYELLTQHWHLKTPNLVISVTGGAKNFALKPRMRKIFSRLIYIAQSKGAWIL  180

synthTRPM8  181   TGGTHYGLMKYIGEVVRDNTISRSSEENIVAIGIAAWGMVSNRDTLIRNCDAEGYFLAQY  240
                  TGGTHYGLMKYIGEVVRDNTISRSSEENIVAIGIAAWGMVSNRDTLIRNCDAEGYFLAQY
humanTRPM8  181   TGGTHYGLMKYIGEVVRDNTISRSSEENIVAIGIAAWGMVSNRDTLIRNCDAEGYFLAQY  240

synthTRPM8  241   LMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTIQDSNYGGKIP  300
                  LMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTIQDSNYGGKIP
humanTRPM8  241   LMDDFTRDPLYILDNNHTHLLLVDNGCHGHPTVEAKLRNQLEKYISERTIQDSNYGGKIP  300

synthTRPM8  301   IVCFAQGGGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVR  360
                  IVCFAQGGGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVR
humanTRPM8  301   IVCFAQGGGKETLKAINTSIKNKIPCVVVEGSGQIADVIASLVEVEDALTSSAVKEKLVR  360

synthTRPM8  361   FLPRTVSRLPEEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTSE  420
                  FLPRTVSRLPEEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTSE
humanTRPM8  361   FLPRTVSRLPEEETESWIKWLKEILECSHLLTVIKMEEAGDEIVSNAISYALYKAFSTSE  420

synthTRPM8  421   QDKDNWNGQLKLLLEWNQLDLANDEIFTNDRRWESADLQEVMFTALIKDRPKFVRLFLEN  480
                  QDKDNWNGQLKLLLEWNQLDLANDEIFTNDRRWESADLQEVMFTALIKDRPKFVRLFLEN
humanTRPM8  421   QDKDNWNGQLKLLLEWNQLDLANDEIFTNDRRWESADLQEVMFTALIKDRPKFVRLFLEN  480

synthTRPM8  481   GLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTFVWKLVANFRRGFRKEDR  540
                  GLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTFVWKLVANFRRGFRKEDR
humanTRPM8  481   GLNLRKFLTHDVLTELFSNHFSTLVYRNLQIAKNSYNDALLTFVWKLVANFRRGFRKEDR  540

synthTRPM8  541   NGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKT  600
                  NGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKT
humanTRPM8  541   NGRDEMDIELHDVSPITRHPLQALFIWAILQNKKELSKVIWEQTRGCTLAALGASKLLKT  600

synthTRPM8  601   LAKVKNDINAAGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELA  660
                  LAKVKNDINAAGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELA
humanTRPM8  601   LAKVKNDINAAGESEELANEYETRAVELFTECYSSDEDLAEQLLVYSCEAWGGSNCLELA  660

synthTRPM8  661   VEATDQHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFIIPLVGCGFVSFRKKPVDKH  720
                  VEATDQHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFIIPLVGCGFVSFRKKPVDKH
humanTRPM8  661   VEATDQHFIAQPGVQNFLSKQWYGEISRDTKNWKIILCLFIIPLVGCGFVSFRKKPVDKH  720

synthTRPM8  721   KKLLWYYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHPPELVLYSLVFVLFC  780
                  KKLLWYYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHPPELVLYSLVFVLFC
humanTRPM8  721   KKLLWYYVAFFTSPFVVFSWNVVFYIAFLLLFAYVLLMDFHSVPHPPELVLYSLVFVLFC  780

synthTRPM8  781   DEVRQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNKSSLYSGRVIFCLDYIIFT  840
                  DEVRQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNKSSLYSGRVIFCLDYIIFT
humanTRPM8  781   DEVRQWYVNGVNYFTDLWNVMDTLGLFYFIAGIVFRLHSSNKSSLYSGRVIFCLDYIIFT  840

synthTRPM8  841   LRLIHIFTVSRNLGPKIIMLQRMLIDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIF  900
                  LRLIHIFTVSRNLGPKIIMLQRMLIDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIF
humanTRPM8  841   LRLIHIFTVSRNLGPKIIMLQRMLIDVFFFLFLFAVWMVAFGVARQGILRQNEQRWRWIF  900

synthTRPM8  901   RSVIYEPYLAMFGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHNLPRFPEWITIPLV  960
                  RSVIYEPYLAMFGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHNLPRFPEWITIPLV
humanTRPM8  901   RSVIYEPYLAMFGQVPSDVDGTTYDFAHCTFTGNESKPLCVELDEHNLPRFPEWITIPLV  960
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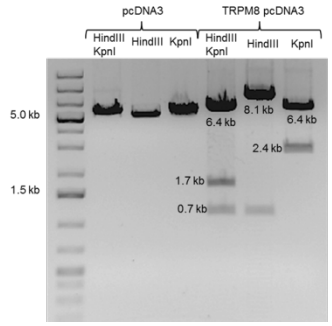
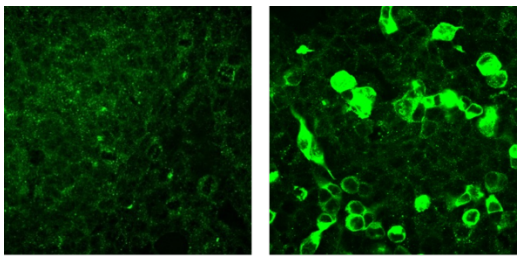
synthTRPM8  961   CIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLNIPFPFIVFAY  1020
                  CIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLNIPFPFIVFAY
humanTRPM8  961   CIYMLSTNILLVNLLVAMFGYTVGTVQENNDQVWKFQRYFLVQEYCSRLNIPFPFIVFAY  1020

synthTRPM8  1021  FYMVVKKCFKCCCKEKNMESSVCCFKNEDNETLAWEGVMKENYLVKINTKANDTSEEMRH  1080
                  FYMVVKKCFKCCCKEKNMESSVCCFKNEDNETLAWEGVMKENYLVKINTKANDTSEEMRH
humanTRPM8  1021  FYMVVKKCFKCCCKEKNMESSVCCFKNEDNETLAWEGVMKENYLVKINTKANDTSEEMRH  1080

synthTRPM8  1081  RFRQLDTKLNDLKGLLKEIANKIK  1104
                  RFRQLDTKLNDLKGLLKEIANKIK
humanTRPM8  1081  RFRQLDTKLNDLKGLLKEIANKIK  1104
```

**(C)** Digestion control of the TRPM8 plasmid (upper panel) and verification of its expression in transfected cell cultures by immunofluorescence (lower panel; cf. Materials and methods section)



pcDNA3 pcDNA3-TRPM8

**HS-SPME-GC/MS method: selected materials**

The method was developed using authentic standards of the eight TRPM8 agonists considered in the present study (Fig. 1). Since no tobacco sample could be obtained that was free of these analytes, method development was carried out matrix-free.

*HS-SPME:* For HS-SPME measurements the coating of the fiber is most important for the extraction of the analytes from the headspace. The examined fiber coatings comprised highly polar (PA), medium polar (CAR/PDMS; PDMS/DVB; DVB/CAR/PDMS) and non-polar (PDMS) materials (cf. Materials and methods section). All fibers could well detect the eight analytes investigated. However, they differed in their adsorption efficiency, which was determined by the integration of the total peak area of the eight analytes. The adsorption efficiencies of the highly polar and non-polar fiber coatings were about 10-times lower when compared to the medium polar coatings (Fig. S2). The medium polar fiber coatings did not significantly differ in their adsorption efficiencies. For quantification the PDMS/DVB fiber was applied because the relative standard deviation (RSD) of the replicates was found lower (i.e. 7.59%) compared to the other two medium polar fiber coatings (16.3 and 24.5%, respectively; cf. Fig. S2). In addition, this was the only fiber without any considerable carry over.

Both the best extraction and incubation times for achieving equilibrium between liquid phase and headspace were determined using the PDMS/DVB fiber. Different extraction times in the range of 5 – 60 min were tested at 60°C and 15 min of incubation time (Fig. S3). The peak area of geraniol, carvone and 7-hydroxycitronellal remained increasing after 60 min of extraction, whereas the other compounds reached equilibrium after 30 min of extraction. Because the extraction time is an important time-limiting factor of the method run, no further timely extension was allowed and 60 min were used as the extraction time of choice. The influence of the incubation time was determined in the range between 5 and 30 min at 60°C and 60 min extraction time (Fig. S4). All eight analytes selected reached the equilibrium after 5 min of incubation. This time was then used for quantification.

After fixing the extraction and incubation time for achieving the best equilibrium between liquid phase and headspace, different incubation temperatures were examined. Analysis was performed by gradually increasing the incubation temperature from 40 to 90°C (Fig. S5). Carvone reached a maximum peak area at 60°C, geraniol and 7-hydroxycitronellal a plateau at 60°C and the other compounds decreased in their peak areas after further heightening of the temperature. Thus 60°C was selected as the temperature of choice.

*GC/MS:* Different GC capillary columns for separation were tested. These were the non-polar HP-5ms and the medium-polar OPTIMA-35ms and DB-17ms columns. All columns achieved a sufficient peak form. When analyzing in SIM mode, the ability for separation of the analytes is of minor interest. However, since menthol and menthone cannot be divided by their

masses they need to be separated on the column. Only the HP-5ms column was sufficient in the separation of these two analytes and was thus used for quantification.
All analytes under consideration were identified by comparison of their mass spectra and retention times to those of authentic standards. Data on retention times, quantifiers and qualifiers are summarized in table S1.

**HS-SPME-GC/MS method: validation**

The GC/MS method was validated for specificity, linearity, precision, limits of detection (LODs), limits of quantification (LOQs), and recovery. To confirm specificity, the retention times and the ratio of qualifier and quantifier ions were compared to those of authentic standards. Calibration curves were analyzed to detect the linear working range for each analyte (table S2). Precision was evaluated by analyzing a standard mix with a concentration of 50 µg/mL five times at one day (intra-day variability) and on two more days three additional times (inter-day variability). The recovery of the analytes in the complex tobacco matrix was examined by spiking additive-free tobacco with 5 µL of the standard mix (concentration of 50 µg/mL). The spiked samples were then processed as described above and the spiked analytes were quantified to determine recovery (table S2). The LOD and LOQ values were determined by means of the German Industrial Norm (DIN) 32645 (see: https://www.beuth.de/de/norm/din-32645/110729574) using the calibration curve method (table S3).
Validation was carried out matrix free, except for the determination of the recovery. There was no shift in retention times and the differences in the ratios of qualifier and quantifier ions for samples and standards were below 10%. The coefficients of determination ($r^2$) for linearity, expect for 7-hydroxycitronellal, were at least 0.99 in the working range of 5 – 300 µg/mL (table S2). As an exception, the determination coefficient ($r^2$) of 7-hydroxycitronellal was found at 0.95 in the working range of 20 – 125 µg/mL. The intra-day precision was determined between 6.4% and 23.7% and the inter-day precision maximum was 15.3% (table S2). All substances could be detected and quantified in the order of nanograms (table S3). The recovery for the eight analytes investigated in the tobacco matrix was between 93.4% and 115% (table S2). The results of method validation are compiled in tables S2 and S3 below.

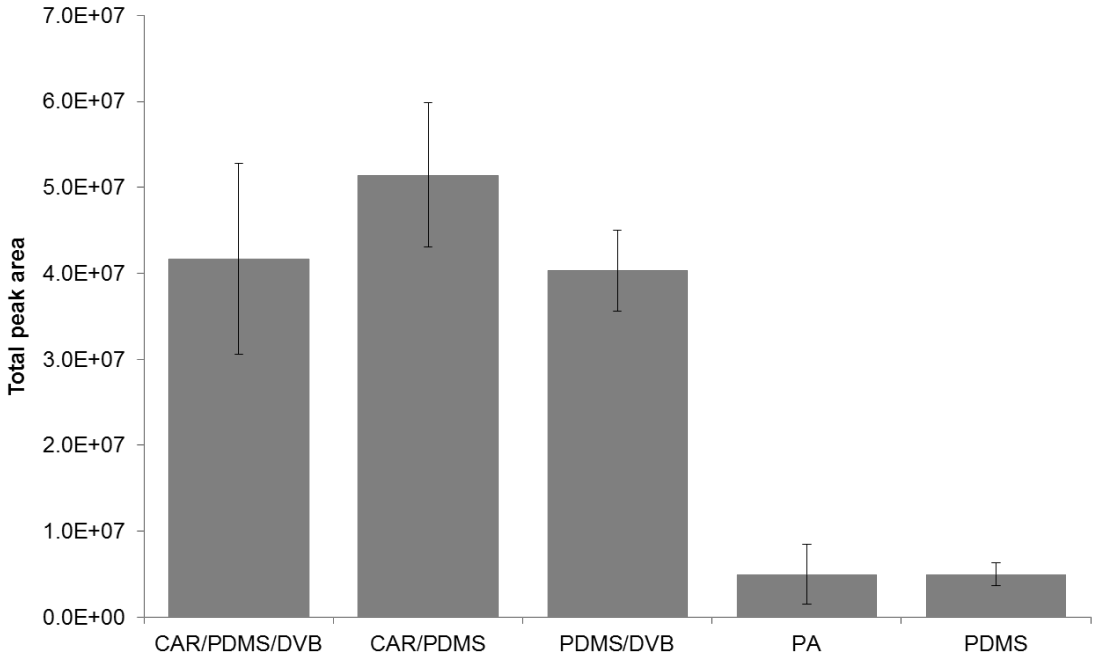



**Fig. S2.** Selection of an appropriate SPME fiber. Total peak areas of the eight TRPM8 agonists extracted from the gas phase via HS-SPME and quantified by means of GC/MS (n=5). Abbreviations: CAR, carboxen; DVB, divinylbenzene; PA, polyacrylate; PMDS, polydimethylsiloxane.

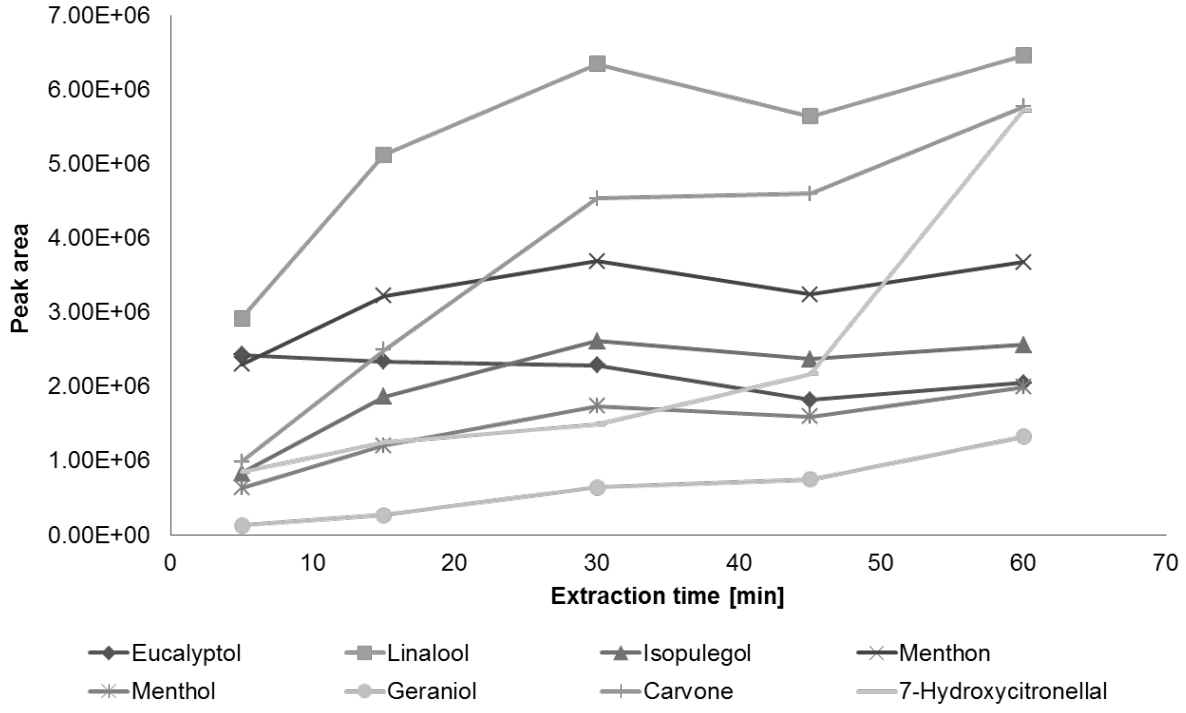



**Fig. S3.** Selection of extraction time. Influence of the extraction time on the efficiency of the fiber (PDMS/DVB) to extract the eight TRPM8 agonists from the gas phase via HS-SPME. The incubation time and temperature were 15 min and 60°C, respectively.

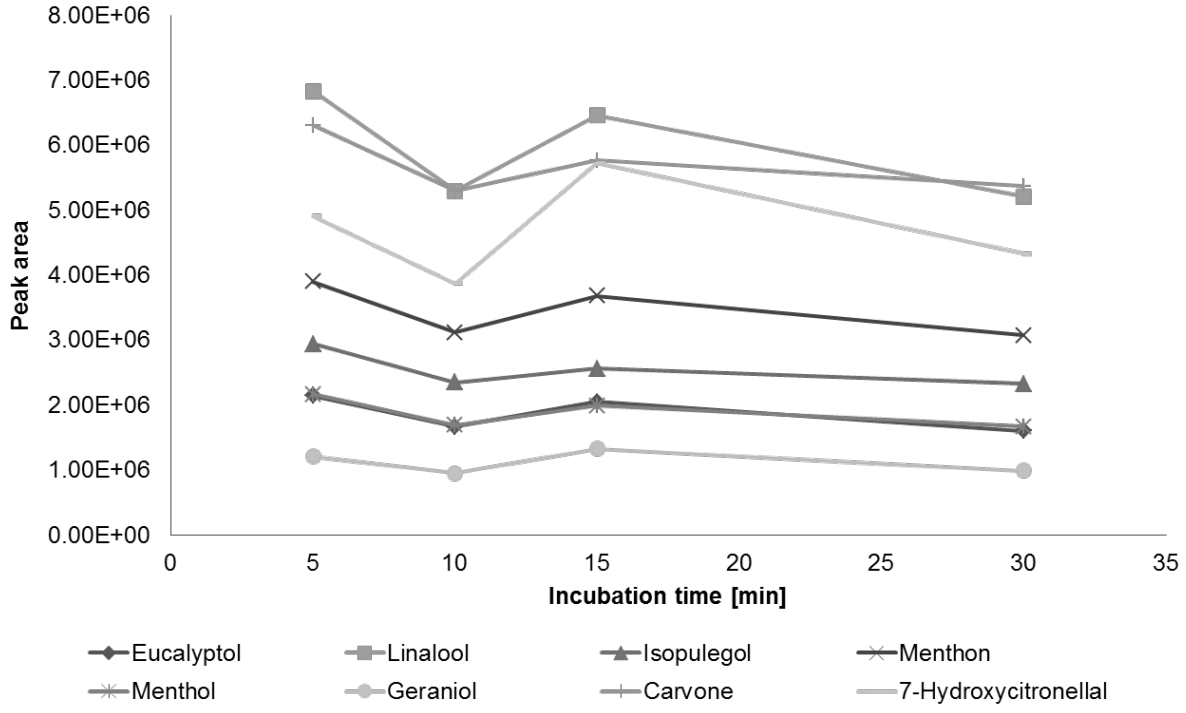



**Fig. S4.** Selection of incubation time. Influence of the incubation time on the efficiency of the fiber (PDMS/DVB) to extract the eight TRPM8 agonists from the gas phase via HS-SPME. The extraction time and incubation temperature were 60 min and 60°C, respectively.

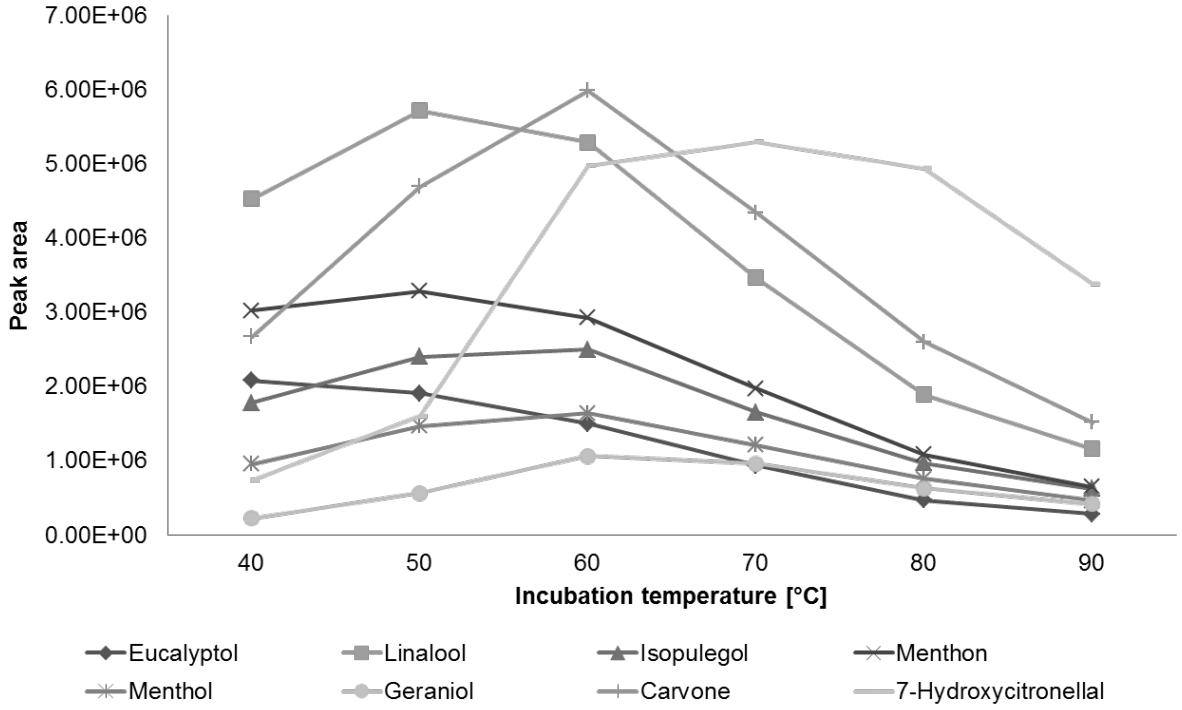



**Fig. S5.** Selection of incubation temperature. Influence of the incubation temperature on the efficiency of the fiber (PDMS/DVB) to extract the eight TRPM8 agonists from the gas phase via HS-SPME. The incubation and extraction time were 15 min and 60 min, respectively.

**Table S1:** Method development: Quantifiers, qualifiers and retention times.

| Compound name | Quantifier | Qualifier | Molecular mass [Da] | Vapor pressure [mmHg at 25°C] | logP | Retention time [min] |
|---|---|---|---|---|---|---|
| Eucalyptol | 154 | 139, 108 | 154.25 | 1.9 | 2.82 | 6.93 |
| Acetophenone-$d_3$ | 123 | 105, 77 | | | | 7.43 |
| Linalool | 93 | 121, 136, 71 | 154.25 | 0.16 | 3.28 | 7.95 |
| Isopulegol | 81 | 121, 136 | 154.25 | 0.005 | 2.92 | 8.71 |
| Menthone | 154 | 69, 112 | 154.25 | 0.3 | 2.63 | 8.83 |
| Neomenthol-$d_4$ | 73 | 99, 127 | | | | 8.97 |
| Menthol-$d_4$ | 73 | 99, 127 | | | | 9.11 |
| **Menthol** | **123** | **138** | **156.27** | **0.0637** | **3.20** | **9.15** |
| Isomenthol-$d_4$ | 73 | 99, 127 | | | | 9.28 |
| Carvone | 82 | 108, 93 | 150.22 | 0.16 | 2.27 | 10.15 |
| Geraniol | 93 | 69, 123 | 154.25 | 0.03 | 3.28 | 10.23 |
| 7-Hydroxycitronellal | 59 | 71, 96 | 172.27 | 0.0058 | 1.54 | 10.67 |

**Table S2:** Validation results: linearity, working range, precision, and recovery rates.

| Compound name | Internal Standard | Working range [µg/mL] | $r^2$ | Intra-day precision [%] | Inter-day precision [%] | Recovery rate [%] |
|---|---|---|---|---|---|---|
| Eucalyptol | Menthol-$d_4$ | 5 – 200 | 0.987 | 6.4 | 4.8 | 98.7 |
| Linalool | Neomenthol-$d_4$ | 5 – 150 | 0.990 | 20.9 | 10.1 | 93.4 |
| Isopulegol | Menthol-$d_4$ | 5 – 300 | 0.997 | 23.7 | 5.2 | 106.0 |
| Menthone | Neomenthol-$d_4$ | 5 – 300 | 0.997 | 11.6 | 8.3 | 100.0 |
| **Menthol** | **Neomenthol-$d_4$** | **5 – 300** | **0.996** | **10.8** | **4.0** | **103.0** |
| Carvone | Menthol-$d_4$ | 5 – 200 | 0.997 | 21.7 | 4.2 | 103.5 |
| Geraniol | Acetophenone-$d_3$ | 5 – 300 | 0.998 | 23.6 | 15.3 | 99.5 |
| 7-Hydroxycitronellal | Acetophenone-$d_3$ | 20 – 125 | 0.947 | 18.5 | 2.5 | 115.0 |

**Table S3:** Limits of detection (LODs) and limits of quantification (LOQs).

| Compound name | $r^2$ | Concentration range [µg/mL] | LOD [µg/mL] | LOD [ng] | LOQ [µg/mL] | LOQ [ng] |
|---|---|---|---|---|---|---|
| Eucalyptol | 0.996 | 0.055 – 0.455 | 0.0418 | 0.209 | 0.119 | 0.595 |
| Linalool | 0.988 | 0.055 – 0.405 | 0.0687 | 0.344 | 0.181 | 0.909 |
| Isopulegol | 0.996 | 0.055 – 0.455 | 0.0414 | 0.207 | 0.121 | 0.604 |
| Menthone | 0.993 | 0.055 – 0.455 | 0.0547 | 0.273 | 0.153 | 0.764 |
| **Menthol** | **0.993** | **0.005 – 0.455** | **0.0511** | **0.256** | **0.152** | **0.760** |
| Carvone | 0.998 | 0.005 – 0.455 | 0.0256 | 0.128 | 0.0821 | 0.411 |
| Geraniol | 0.965 | 3 – 10 | 3.31 | 16.54 | 9.50 | 47.51 |
| 7-Hydroxycitronellal | 0.968 | 2 – 10 | 2.93 | 14.65 | 8.97 | 44.87 |

**Table S4:** Contents of TRPM8 agonists in menthol cigarettes [µg/cigarette]

| Brand | Linalool | **Menthol** | Menthone | Carvone | Eucalyptol | Isopulegol |
|---|---|---|---|---|---|---|
| A | n.d. | 2660 | 1.85 | 0.37 | 0.15 | 2.01 |
| B | 4.05 | 4190 | 13.9 | 16.0 | 2.60 | 5.93 |
| C | 0.67 | 2890 | 1.38 | n.d. | n.d. | 0.73 |
| D | 3.68 | 516 | 37.8 | 270 | 10.6 | 6.00 |

n.d.: not detected

**Table S5:** Contents of TRPM8 agonists in American blend cigarettes [ng/cigarette]

| Brand | Linalool | **Menthol** | Menthone | Carvone |
|---|---|---|---|---|
| A | 141 | 100 | n.d. | 77.7 |
| B | 104 | 445 | 35.9 | 58.9 |
| C | 98.2 | 115 | 37.9 | 88.3 |
| D | 142 | 58.2 | n.d. | n.d. |

n.d.: not detected

**Table S6:** Contents of TRPM8 agonists in additive-free cigarettes [ng/cigarette]

| Brand | Linalool | **Menthol** | Menthone | Carvone | Eucalyptol | Geraniol |
|---|---|---|---|---|---|---|
| A | 105 | 71.5 | n.d. | 71.6 | n.d. | 92.1 |
| B | 229 | 256 | 103 | 76.9 | n.d. | 67.7 |
| C | 75.8 | 36.2 | 22.6 | 36.2 | 40.5 | n.d. |
| D | 168 | 23.4 | 40.4 | n.d. | n.d. | 125 |

n.d.: not detected

**Table S7:** Contents of TRPM8 agonists in light cigarettes [ng/cigarette]

| Brand | Linalool | **Menthol** | Menthone | Carvone | Geraniol | 7-Hydroxycitronellal |
|---|---|---|---|---|---|---|
| A | 166 | 3630 | 53.3 | 46.4 | 98.8 | 105 |
| B | 261 | 646 | 153 | 132 | 206 | n.d. |
| C | 138 | 140 | n.d. | 84.0 | 146 | 190 |
| D | 155 | 121 | n.d. | 43.2 | 87.4 | 50.4 |

n.d.: not detected

# 4 Discussion

*“There are epidemics that are new and frighten us and there are health hazards, to which we got used although they are still from epidemic proportion.”*

Dietmar Jazbinsek, 2021

Tobacco is the only legal available consumer product that is estimated to kill half of its users, when consumed as recommended (Borland, 2003). It is the most preventable cause of disease and death worldwide (Mauer-Stender et al., 2019). In Germany tobacco use causes about 127.000 deaths per annum, a number that represents 13% of all deaths in this country (Kotz et al., 2018). Also, economically tobacco consumption is a social problem, the received taxes from tobacco sales are far from the costs produced for public health services and economics need to repair the damage induced. However, the government should not strictly interfere with the kind of vices people would engage with, therefore the prohibition of tobacco consumption is neither realistic nor to aspire. It has been well experienced in the case of other targets of national prohibition, such as the alcohol prohibition between 1920-1933 in the USA, that such restrictions always lead to exuberant criminalisation of large parts of the population. The positive effects of prohibition are rather little though, mainly due to the failure to effectively prevent consumption of sought-after consumer goods (Hall, 2010; Chrystoja et al., 2020).

A more promising approach to control the use of noxious and addictive substances is to reduce its demand in the population. In 2004 a tobacco control scale (TCS) was implemented in 30 European countries to quantify tobacco control measures in the member states. From the very early beginning after the implementation of the TCS, Germany always occupies one of the lowest rankings on the scale. In 2019 Germany was placed even at the undermost position of this scale. Responding to this embarrassing situation, the German Cancer Research Centre (DKFZ) presented a strategy for the path to reach an almost tobacco-free Germany by the year 2040 (Graen and Schaller, 2021). The goal is to achieve numbers of no more than 5% of adults and less than 2% of adolescents who still might consume tobacco products or e-cigarettes in Germany at this date (Graen and Schaller, 2021). Even though Germany’s ranking in the TCS seems unjustifiable, its relative numbers of current smokers represent the average of the numbers which are known from the other 29 European countries that signed the TCS. Not to be sneezed at, the level of current smokers is constantly decreasing in Germany. To date, 23% of the German population is smoking regularly (Eurobarometer, 2021), whereas this number has been 25% in 2017 (Kotz et al., 2018) and 26% in 2012 (Eurobarometer, 2012).

With the TPD 2014/40/EU all Member States are required to implement strategies to reduce the demand for and supply of tobacco products. In that Directive a special focus is set on additives used for tobacco products. The additives used shall not enhance the toxicity, addictiveness and attractiveness of the product. In that respect also characterising flavours for cigarettes and RYO tobacco will be prohibited.

However, it remains unclear how to reliably characterise and measure the influence of additives on the overall toxicity, addictiveness and attractiveness of tobacco products. The aim of this thesis was to provide reliable methods to examine such effects attributable to the tobacco additives. To this end, the following different analytical approaches have been established and used:

- A screening study was performed to address decomposition products of complex tobacco additives.
- By means of a targeted screening approach key substances of strawberry flavour in tobacco products were identified and estimated.
- Enantioselective studies of known tobacco flavourings were performed.
- A method was developed and validated to quantify the menthol contents in cigarettes.
- A bioassay has been established and subsequently used to determine the biologically active levels of menthol via its TRPM8 agonism.

## 4.1 Smoke simulation studies of tobacco additives

To evaluate the smoke chemistry of tobacco additives the preferred method employed by the tobacco industry is to use so-called smoking machine protocols (Sabbert et al., 2019). These standardised protocols were developed to measure the tar contents of cigarette smoke in a reliable and reproducible way. According to the Directive 2014/40/EU the manufacturers are required to inform the authorities whether the additives used can increase the addictive or toxicological potential of cigarettes. Therefore it is not sufficient to compare tar yields of cigarette smoke only. In addition, a range of certain smoke constituents need to be evaluated and assessed as well.

There are some caveats to be considered in the case of smoking machine measurements when it comes to the screening of smoke constituents originating from tobacco additives. The collection (adsorption) of volatile organic compounds generated by the machine smoking process on glass fibre filters is highly prone to compound loss. Bush et al. (2012) pointed to the differences between the profiles of smoke components generated by either an on-line coupled smoke generator or a conventional smoking

machine. In their study, the use of an on-line coupled smoke generator resulted in about four times higher yields of carbonylic compounds when compared to the conventional smoking machine. Besides this application of the latter method reveals difficult in the analysis of certain cigarette components. Moreover, smoking machine protocols are time consuming and thus not suitable for a first screening of follow-up products of different additives in the smoke generated. As already mentioned in the introductory part (section 1.3), about 600 different tobacco additives are known today, awaiting for its further analysis with regard to their addictive and toxicological potential which they might add on top of the deleterious effects of cigarette smoke itself.

To detect even small changes in the composition and toxicity of cigarette smoke high numbers of control and experimental preparations are required for comparison (Paumgartten et al., 2017). On-line oxidative py-GC/MS not only enables high sample throughput but also can simulate the heating conditions of the burning cigarette in a perfect way. Further, the generated volatile compounds can be directly subjected into the GC/MS system, thus avoiding compound loss due to the use of filter materials. Not least, another advantage of the pyrolysis approach is the possibility to analyse single substances and their follow-up products easily.

The European TPD (EU, 2014) mainly focusses on additives rather than the toxicological potential of tobacco itself. Therefore pyrolysis profiles of the additives should be generated both in the absence and presence of the tobacco matrix. Based on these profiles the toxicological and addictive potential of the additives shall be assessed and estimated.

In the current work an untargeted py-GC/MS-based approach has been established and performed in order to get insights on the broad variety and identity of the chemical species formed upon pyrolysis. Using this setup, however, not all relevant analytes might become detectable. As a general problem in the analysis of complex organic matter, peaks can be masked by more dominant (quantitatively predominant) compounds. Such an overlapping of peaks would result in a reduced number of library matches, therefore only matches with a probability of >85% and a peak area of >0.1% of the total peak area were used for further consideration.

### 4.1.1 Toxic smoke constituents from tobacco additives

Since cigarette smoke is inherently harmful, demonstrating the toxicological potential of additives in this matrix is quite challenging (Paumgartten et al., 2017). Tobacco itself consists to a large portion (>30%) of fibre-like materials such as cellulose, hemi-cellulose and pectin (Seeman et al., 2002). In 2002, Seeman and coworkers suggested that these

polymers may have a greater effect on the generation of some Hoffmann analytes than smaller molecules like sugars or semi-volatile compounds (Seeman et al., 2002). On the other hand, increases in the levels of formaldehyde, acrolein, cadmium (Cd), palladium (Pd), hydrogen cyanide (HCN) and resorcinol in cigarette smoke could be detected upon supplementation of tobacco with additives (Paumgartten et al., 2017). Most of the conducted studies were based on the detection of the 44 Hoffmann analytes. Certainly, these compounds represent only a small fraction of all toxicants present in cigarette smoke (Paumgartten et al., 2017).

By means of the py-GC/MS method established, 72 different compounds with toxic potential could be detected in the frame of our studies. Among these five have not been reported in connection to tobacco or tobacco smoke before (Rodgeman and Perfetti, 2013). This observation provides further evidence that tobacco additives indeed contribute to the formation of additional toxic compounds that are unlikely to occur in the smoke of additive-free tobacco. An even more complex objective of the European TPD is the prohibition of tobacco additives that contribute or even enhance the addictiveness or attractiveness of the product. Our experimental work delivered the proof that besides the already mentioned additional toxicants other bioactive substances also emerged in the smoke of tobacco that has been replenished with certain flavouring additives.

### 4.1.2 Smoke constituents originating from tobacco additives that might affect the addictiveness of the product

Sugars influence not only the pH-value of tobacco smoke and thus contribute to a smoother feeling of the smoke, they also lead to a sweeter taste upon temperature-induced caramelisation (Talhout et al., 2006). Both effects are likely to heighten the acceptance of cigarette smoke (Talhout et al., 2006).

Besides these taste related effects, the addictive potential of cigarette smoke is not solely a matter of nicotine uptake. Other smoke constituents than nicotine may also contribute to the addictiveness of tobacco products. Pyrolysis of sugars leads to the formation of acetaldehyde. Most likely, condensation products of acetaldehyde and certain biogenic amines can inhibit the enzymes that belong to the group of monoamine oxidases (MAO) (Talhout et al., 2006). MAO is an enzyme family, consisting of $MAO_A$ and $MAO_B$ in humans that contribute to the metabolic conversion (inactivation) of neurotransmitters such as dopamine, noradrenaline or serotonin. There is convincing experimental evidence to suggest that increased levels of these neurotransmitters in the brain contribute to the addictiveness of cigarette smoke, an observation that in turn might result from the inhibition of MAO (Benowitz, 2010).

The pyrolytic studies conducted in the frame this thesis work (described in section 3.1) could demonstrate that acetaldehyde is regularly formed through pyrolysis of sugars (polysaccharides). In the literature, however, the influence of additives on the levels of acetaldehyde in cigarette smoke has been controversially discussed (Thalhout et al., 2006).

For the qualitative assessment of the follow-up products of tobacco additives, the pyrolysis approach applied in this work can be a helpful tool, too. However, to determine the contribution of individual tobacco additives to the overall toxicity of the tobacco matrix, quantitative analyses are mandatory.

### 4.1.3 Formation of PAHs during pyrolysis of cocoa and tobacco

In a semi-quantitative approach, the occurrence of 20 different polycyclic aromatic hydrocarbons (PAHs) during pyrolysis of tobacco with or without cocoa has been monitored. The findings obtained pointed to the assumption that the amounts of PAHs formed in tobacco smoke are not significantly affected by the addition of cocoa. This result is in line with the findings presented by Seeman and coworkers in 2002, who reported that the formation of Hoffmann analytes is not being altered by the addition of cocoa to complex matrixes such as tobacco (Seeman et al., 2002).

Running the on-line pyrolysis approach under inert conditions at 1000°C, however, resulted in higher amounts of the highly volatile PAHs naphthalene and acenaphthylene when compared to the smoking machine tobacco experiments reported in the literature (Forhand et al., 2000; Tarrant et al., 2009; Shi et al., 2015). This outcome could result from compound loss during the conduct of the smoking machine protocol. On the other hand, the entire sample setup procedure during the pyrolysis experiments performed (see section 3.1) could contribute to an enhanced transition of the PAH volatiles into the gas phase. For the less volatile PAHs the levels measured in our experiments were highly consistent with previous reports on their formation patterns (Forhand et al., 2000; Tarrant et al., 2009; Shi et al., 2015).

## 4.2 Chemical analyses of characterising flavours used in cigarettes

According to the European Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) sweet and tasteful tobacco products may target in particular children and adolescents (SCENIHR, 2010). Hoffman et al. (2016) reviewed data about the flavour preferences of the young and the adults and came to the conclusion that minors are more attracted by sweet and fruity flavourings when compared to adults. It is

known that the majority of young people's first smoke experiences rely on flavoured tobacco products (Ambrose et al., 2015). Although the proportion of adolescent smokers is continuously decreasing, most people still start smoking in this age group (US Department of Health and Human Services, 2012).

Despite the ban of characteristic flavours in cigarettes and RYO tobacco, flavouring of e-cigarette liquids, cigars, hookah (waterpipe) and smokeless tobacco products still persists. It seems obvious that these products can promote the initiation of tobacco use in young people. On the other hand, introducing flavourings that are usually preferred by adults might stimulate product switching or dual use among adult smokers (Hoffman et al., 2016). A recent study indicated that the tobacco product flavour ban in San Francisco led to a decrease in the prevalence of the total flavoured tobacco use (Yang et al., 2020). The application of sweetening agents that are unable to create a characteristic flavour is still allowed for all tobacco products though. Thalhout et al. (2006) pointed out in their study that the sweet taste and pleasant smell of caramelised sugars seem attractive especially to adolescents. So, adding sugars to tobacco not only modifies the flavour of a cigarette, they also contribute to a smoother feeling of the inhaled smoke (Thalhout et al., 2006).

Since strawberry is among the most popular fruits worldwide (Ulrich et al., 2018), many tobacco products exuding a strawberry flavour can be found on the market. To identify key compounds mainly responsible for the characteristic flavour of strawberry reveals quite challenging. Naturally strawberry flavour consists of about 360 different compounds including esters, alcohols, ketones and aldehydes (Latrasse, 1991). The exact identities and the concentrations of these analytes can vary strongly based on cultivar and the stage of ripeness of the strawberries (Yan et al., 2018). Many of these compounds will be detectable in other fruits as well. For the detection of the relevant flavour determining substances, it is important to consider that not all of the detected volatile compounds would exert an influence on the sensory profile of the flavour under consideration. In this regard, the so-called odour active value (OAV, that is, the ratio between concentration and sensory threshold of a particular compound) will be important. Compounds with an OAV less than 1 are unlikely to contribute to a particular flavouring note (Yan et al., 2018). Furthermore, the likely presence of chiral components and their individual enantiomers would make it even more difficult to identify key substances responsible for fruity flavours such as strawberry. The individual enantiomers of a chiral compound regularly have different physiological (Behrendt et al., 2004) and organoleptic properties (Lytra et al., 2014).

### 4.2.1 Targeted screening for marker compounds of strawberry flavour in cigarettes

The aim of the study described in section 3.2 was to define chemical compounds indicative for strawberry flavour that could be present in tobacco products. These marker compounds could then be used for an analytical screening of the additives used in tobacco products to verify or falsify the data submitted by manufacturers and importers as annual reports to the national authorities. The compounds found and assigned to be relevant to create strawberry flavour, nicely mirrored the strawberry flavour compounds that have been described in literature (Ayala-Zavala et al., 2004; De Boishebert et al., 2006; Zhang et al., 2009; Dong et al., 2013). However, some of these compounds were detected in other fruity flavoured cigarettes and fine cut as well and had thus to be excluded. In addition, compounds that are known to be present in the mainstream smoke of cigarettes are also not qualified to serve as markers for the identification of strawberry flavour in tobacco products. For instance, this refers to 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (furaneol) which has been also described as key substance for natural strawberry flavour in the literature (Schwab, 2013).

### 4.2.2 Enantioselective characterisation of tobacco flavourings

The distinction between enantiomers should be kept in mind when it comes to the identification of the composition of characterising flavours. As an example, the odour of (*S*)-limonene is described as terpenic/herbal and would not relate to a fruity flavour. On the other hand, (*R*)-limonene smells like citrus and therefore can be frequently found in fruity flavours. Monitoring the enantiomeric ratio (ratio of the mirror image isomers) of the chiral compounds in the flavouring mixtures is of importance because of significant differences in their physiological properties. For instance, the cold-menthol receptor TRPM8 can mask irritant properties of tobacco smoke in bronchial epithelia when being activated by some sorts of agonists. With regard to menthol, this receptor will be predominantly activated by the natural occurring (1*R,*3*R,*4*S*)-(–)-menthol, when compared to its enantiomer, the (1*R,*3*R,*4*S*)-(+)-menthol. Other chiral compounds such linalool are also present in strawberry flavour and capable of activating the TRPM8 receptor (Behrendt et al., 2004). The physiological differences of the enantiomers of such compounds have yet not been addressed and remain unclear. Since enzymatically catalysed biosynthesis routes of volatiles in plants usually occur via stereoselective or even stereospecific reaction pathways, many natural flavour compounds emerge with high enantiomeric excess (Larkov et al., 2008). Therefore the enantiomeric ratio often reveals specific for the particular biological source (König and Hochmuth, 2004). This characteristic can be used for authenticity testing. Similar as in the food industry tobacco

manufacturers could claim that only natural sources were used to obtain the additives needed. In such cases the authenticity of the flavouring mixture would be of interest. Not all chiral compounds are usable for the purpose of authenticity testing though. So, the enantiomeric ratio of a compound might be sensitive to certain environmental conditions. Different methods of processing may have different impacts on the stability of a certain enantiomer. For instance, an increase in the level of (*R*)-linalool has been detected after freeze drying of strawberries. This observation has been reported in the study described in section 3.2. Besides temperature variations, acidic and alkaline conditions are also likely to affect the enantiomeric ratio of certain chiral compounds. For that reason, the proof of authenticity should always rely only on such compounds that are stable in their enantiomeric composition, something that need to be experimentally verified beforehand.

## 4.3 Determination of the physiological active levels of menthol

Menthol use in cigarettes is not only favoured because of flavouring reasons. Caused by the ability to mask irritating effects of the cigarette smoke and its positive sensory mint-like effects menthol is the most frequently used tobacco additive. It is assumed that about 25% of all cigarettes sold contain menthol, even brands that claim to be non-mentholated (Kopa and Pawliczak, 2020). For the implementation of the European TPD it becomes important to investigate whether the levels of menthol used in these supposedly non-mentholated products are high enough to create cooling sensations.

### 4.3.1 Quantitative analysis of menthol in cigarettes

Quantitative measurements in complex matrixes such as tobacco or cigarette smoke are quite challenging. By applying a fast and sensitive SPME-GC/MS method, however, the analysis and quantification of menthol in such complex matrixes became possible. With regard to quantification of analytes, the matrix effects and thus the distribution of volatiles between the headspace and the SPME fibre need to be taken into account. At the beginning it is further not clear if the tobacco matrix itself contains some amounts of the analytes under consideration. In that case the tobacco matrix would contributes to the signals detected. Therefore, standard addition would be the method of choice for an HS-SPME-GC/MS-based quantification. Each sample is measured either unspiked or spiked to follow elevating calibration points and to create a linear relationship (regression) between the levels of the analyte and the signals produced by the respective sample. To reach higher sensitivity of the method, the MS should monitor the chromatogram in the

selected ion monitoring (SIM)/scan modus (Jenden and Cho, 1979). The SIM modus means that only characteristic ions of the analytes under consideration will be monitored, leading to a higher sensitivity of the method. The quantification relies on a single ion (quantifier ion) that should not only be characteristic for the substance but also highly abundant. Further, two or three additional qualifier ions should be monitored to enable an unambiguous qualification (identification) of the analyte. The scan modus provides total ion chromatograms (TIC) that do not exclusively refer to the analytes under consideration. To obtain reliable results, the validation of the analytical method therefore becomes crucial and indispensable.

Besides menthol other monoterpenes might be also capable of activating the cold-menthol receptor, namely: geraniol, linalool, eucalyptol, isopulegol, 7-hydroxycitronellal, and carvone (Behrendt et al., 2004; Bharate and Bharate, 2012). Applying the method that has been developed and validated in the frame of this thesis work, the following types of cigarettes were analysed for their contents of menthol and all the other TRPM8 agonists mentioned above: American blend, additive-free, light and mentholated cigarettes. Moreover, the dried leaves of self-grown smoking tobacco were analysed with regard to the levels of the TRPM8 agonists contained. It has been already demonstrated before that virgin tobacco leaves only contain minor amounts of menthol. Merckel et al. (2006) quantified the natural contents of menthol in self-grown tobacco and provided a number of 0.02 μg per gram. In addition, the authors also detected higher contents of menthol in supposedly non-mentholated cigarettes when compared to raw tobacco. It thus seems likely that complex additive mixtures, used to replenish cigarette tobacco, may have contributed to an increased amount of menthol in these kinds of products. Based on this assumption, we decided to look for the presence of TRPM8 agonists in cocoa and liquorice, both of which are used as complex additive mixtures to refine cigarette tobacco prior to sale.

In the study conducted (described in section 3.3) the estimated contents of menthol in self-grown tobacco leaves were comparable to the levels found in the American blend, additive-free and some but not all light cigarettes tested. This result may indicate that no further menthol was added during production of these cigarettes. However, the mean contents of menthol in the different light cigarette brands tested in our study led to the assumption that menthol might have well been added during production in some cases. In principle, higher menthol levels in some cases could also be due to some kind of contamination during the processing of these particular cigarettes. Since it is well known that nicotine and tar levels will become diluted in the mainstream smoke of light cigarettes due to ventilation holes in the filter, it is more likely that menthol was indeed

artificially added to compensate for its loss during smoking (Jaccard et al., 2019). The results obtained also show that the monoterpenes under consideration can be brought into the tobacco through complex additive mixtures. However, since the contents of TRPM8 agonists detected in cocoa and liquorice were comparatively low, the overall effects of these physiologically active compounds are assumed to be rather low.

In 2017, Krüsemann and co-workers determined the sensory threshold of menthol in cigarettes. This threshold is of importance when characterising flavours in cigarettes will be prohibited by the European TPD. In their sensory experiment, conducted with a panel of non-smoking volunteers, the sensory threshold has been estimated to be at 1.8 mg menthol per gram tobacco (0.18%) (Krüsemann et al., 2017). Based on an average tobacco content of 700 mg per cigarette the sensory threshold for menthol would be at 1.3 mg per cigarette. The amounts of menthol measured in our study in the case of American blend, additive-free as well as light cigarettes were well below this threshold level. Only some mentholated cigarette brands revealed menthol levels of up to 4.19 mg/cigarette that would be high enough to create a characterising flavour. These numbers were nicely comparable with the menthol contents reported in the literature. So, Ai and co-workers detected an average of 4.75 mg menthol per cigarette in products advertised as being mentholated (Ai et al., 2016). Jaccard et al. (2019) determined the levels of menthol in 33 different cigarette brands including capsule cigarettes, and reported numbers between 1.17 and 21.6 mg/cigarette.

Artificially mentholated cigarettes have thus been prohibited by means of the European TPD for their suitability to create a characterising mint-like flavour (EU, 2014). The remaining issue associates with the question of whether or not menthol contents in cigarettes, although well below the sensory threshold, would still be sufficient enough to activate the cold-menthol receptor. If so, such products, though not being labelled as menthol cigarettes, might still contribute to the enhancement of inhalation of cigarette smoke and thus facilitating the absorption of nicotine in the lungs.

### 4.3.2 Assessment of menthol levels required to activate TRPM8

To estimate the minimum levels of menthol that are sufficient to activate the TRPM8 receptor, a cell-based bioassay has been developed (described in section 3.3). Human TRPM8-cDNA was co-transfected with cyano fluorescence protein-nuclear marker (CFP-nuc) into human embryonic kindney (HEK) 293 cells. CFP-nuc was used to visually confirm the successful transfection of TRPM8. The genetically modified cells were then loaded with the cell permeable calcium ion indicator Fluo-4 AM. Upon activation of TRPM8 (by agonists such as menthol) this cation channel will be opened, thereby

promoting a calcium influx into the cytosol of the cells. Depending on the intracellular $Ca^{2+}$ level, Fluo-4 AM will then be converted into to a fluorescent dye. Altogether this setup allows the monitoring of TRPM8 activation (chemically mediated agonism) via online fluorescence microscopy (Figure 4-1).

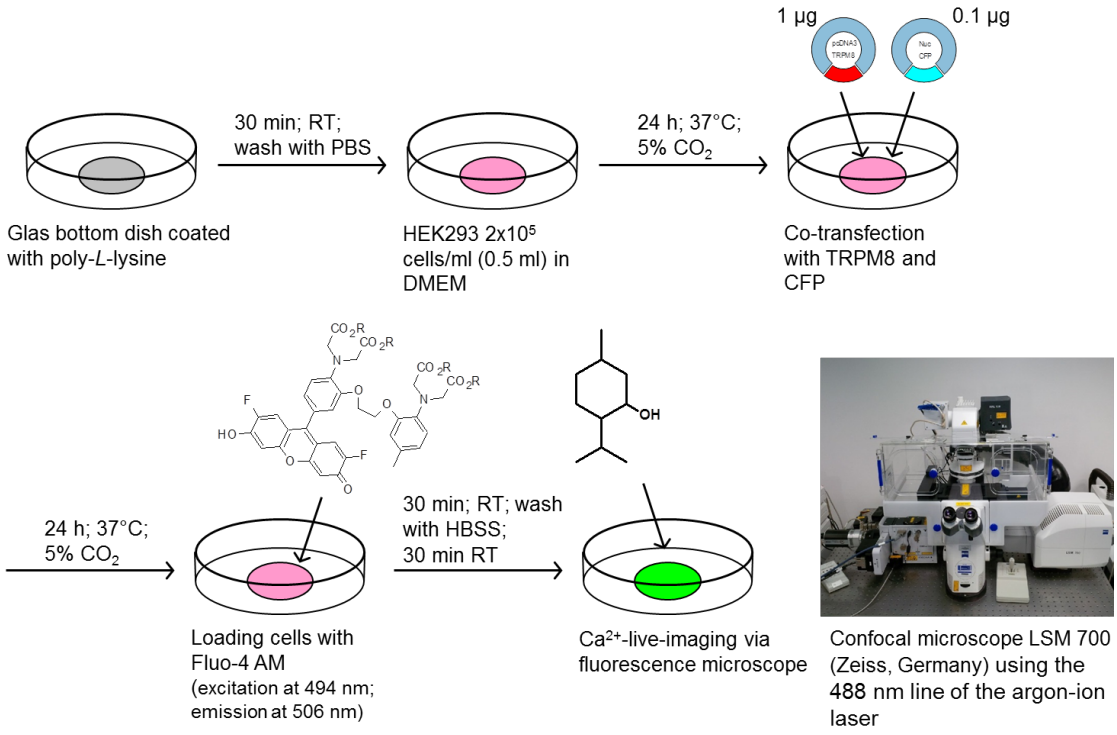



**Figure 4-1 Experimental setup of the $Ca^{2+}$ Imaging method.**
Abbreviations used: CFP, Cyan Fluorescent Protein, DMEM, Dulbecco's Modified Eagle Medium, HBSS, Hanks' Balanced Salt Solution, HEK, Human Embryonic Kidney (cells), PBS, Phosphate Buffered Saline, RT, Room Temperature, TRPM8, Transient Receptor Potential Cation Channel Subfamily Melastatin Member 8.

Although no clear dose response relationship could be observed, the $Ca^{2+}$ influx measured after application of 0.025 µM menthol was only vanishingly small when compared to higher menthol concentrations. Therefore, it was assumed that at least 0.1 µM of menthol would be needed to promote the activation (opening) of the TRPM8 receptor in HEK293 cells in culture. Willis et al. (2011) reported a minimum level of menthol required to activate the cold-menthol receptor *in vivo* at about 0.65 µM. This number led to the estimation that the relevant concentration of menthol in tobacco smoke, required to activate the cold-menthol receptor, lies somewhere between 0.1 µM and 0.65 µM. According to the ISO smoking regime, the total smoking volume of one cigarette is about 280 ml (8 puffs, 35 ml each) (ISO 3308, 2012). Given these numbers, the concentrations of 0.1 µM (= 15.6 µg/L) and 0.65 µM (= 101 µg/L) would be aequivalent to a level of menthol in the smoke of one cigarette between 4.4 µg and 28.4 µg.

To experimentally address the question of how much menthol in one cigarette would be sufficient to induce a “cooling effect” in the bronchial epithelia, it becomes important to consider that menthol is a highly volatile flavouring compound that would be subject to an equilibration between paper, filter and packaging. In addition, little is known about the stability of menthol and its transition into the mainstream smoke during smoking. In 2014, a menthol transfer of 30% was measured by MacGregor and colleagues (MacGregor et al., 2014). This was in line with the assumption made in earlier studies, e.g., the one conducted by Bozinski and co-workers in 1972. A newer study of Jaccard et al. in 2019 tested different ISO smoking regimes with regard to the menthol transfer. The application of these ISO protocols resulted in menthol transfer rates between 1% and 17%, however, the so-called “intense” ISO protocol led to a number of up to 40% (Jaccard et al., 2019). Considering the earlier publication that reported a menthol transfer from the cigarette into the mainstream smoke of about 30% (MacGregor et al., 2014), along with the above-mentioned minimum level of menthol in the smoke required to activate the cold-menthol receptor (somewhere between 4.4 µg and 28.4 µg), contents of 15 to 95 µg menthol per cigarette would be calculated. Assuming a level somewhere in between, we suggested a minimum menthol content of about 50 µg/cigarette to be likely to lead to a detectable effect of sensation in the lungs of smokers. Lower amounts might still result in a partial activation of the TRPM8 receptor, yet receptor channel opening would be rather unlikely when menthol contents drop below the level of 4 µg per cigarette.

The results of our menthol studies (described in section 3.2) reveal that the highest menthol content found in non-mentholated cigarettes was about 3.6 µg per cigarette. Therefore, the non-mentholated cigarettes investigated in our studies are unlikely to be capable of activating the TRPM8 receptor. On the other hand, previous experiments reported in the literature demonstrated that non-mentholated cigarettes with menthol contents higher than 4 µg/cigarette actually do exist (Ai et al., 2016; Richter et al., 2016).

In summary, mentholation of cigarettes that yield menthol amounts in the product below the sensory threshold of 1.3 mg/cigarette (see. 4.3.1., Krüsemann et al., 2017) would not underlie the TPD with regard to the definition of a so-called characterising flavour, yet might well be sufficient enough to induce a physiological response (cooling effect, already starting at 50 µg/cigarette). Nevertheless, these products most likely would also contribute to the enhancement of inhalation (addictiveness) and to a gain in the attractiveness of the resulting tobacco product. Therefore they should be targeted by an appropriate regulation.

## 4.4 On the stage of the implementation of the EU Tobacco Products Directive 2014/40/EU

The relaunched TPD was entered into force on May 19, 2014, and from May 20, 2016, it became applicable. The European Commission has developed several legislative acts to facilitate the implementation of the TPD. For example, the commission implementation (EU) 2015/2186 defines the format for the submission of information on tobacco products. In accordance with this format, manufacturers and importers submit data on additives, emissions and toxicology of their tobacco products to the EU Common Entry Gate (EU-CEG) (European Commission, 2015). In the case of certain additives enhanced reporting obligations do exist. These additives are mentioned in the commission implementation decision (EU) 2016/787 (European Commission, 2016a).

However, only little information on priority additives has been established so far. For 14 out of the 15 priority additives a consortium of 12 tobacco manufacturers submitted reports to the European Commission. Enhanced reporting for the compound diacetyl (2,3-butanedione) is still lacking. Since the leading tobacco companies do not use this particular additive, diacetyl was excluded from the list of priority testing by manufactures. The transfer of additives into the tobacco mainstream smoke was monitored by smoking machine measurements, whereas its toxicity has been analysed via *in vitro* assays only (Stabbert et al., 2019). On the other hand, the ability of tobacco additives to enhance the addictiveness and the attractiveness of the product was investigated in clinical studies (McEvan et al., 2019). In addition, a sensory method was developed by the tobacco companies to prove if the priority additives would be capable of creating a characterising flavour in cigarettes (Simms et al., 2019). The independent review panel of experts of the Joint Action on Tobacco Control (JATC), however, came to the conclusion that the reports submitted by the tobacco industry consortium provided only little usable information. They criticised that previously identified pyrolysis products have not been discussed and toxicologically assessed in the reports submitted. For instance, inhalation studies were not performed at all to support toxicological evaluation. The applied statistical approaches were highly prone to produce false negative results (Bolling et al., 2022). On the other hand, there were neither appropriate guidelines provided by the EU Member States on how to monitor the priority additives, nor defined or specified information available on the data that were supposed to be reported.

The JATC expert panel and the Application Report of the European Commission criticise, that based on the limited data submitted by the industry for these additives, their contribution to the overall toxicity and addictiveness of tobacco products could not be devitalised. In consequence, a more reliable approach would be required to assess

tobacco ingredients and additives for their chemical and physiological properties, in either case burnt or unburnt (European Commission, 2021; Havermans et al., 2022).

For the irretrievable prohibition of characterising flavours in tobacco products a four-year phase-out period had been agreed upon. Accordingly, starting from May 2020 no characterising flavours (including menthol) were allowed in the EU anymore. The document provided by the EU Commission as Implementation Regulation (EU) 2016/779 (European Commission, 2016b) elaborates on the procedures of how to determine characteristic flavours. This Regulation was implemented by the Independent Advisory Penal (IAP) on characterising flavours in tobacco products, as described in the Commission Implementation Decision (EU) 2016/786 (European Commission, 2016c). The IAP helps to decide whether or not a tobacco product is furnished with a characterising flavour. However, the proposed methods are very time consuming and it has to be assessed how the EU-CEG and the IAP could work in a more effective and robust way (European Commission, 2021).

In line with Article 28 of Directive 2014/40/EU, new scientific and technological developments need to be considered in the Application Report of the European Commission. Therefore the appearance of new products on the market, such as, e.g., flavour capsules for self-administration and menthol cards for cigarette flavouring (Figure 4-2), should be urgently addressed in the future. It can be assumed that such newly generated products will be on sale mainly for the reason to bypass the ban of characterising flavours in tobacco products, which includes capsule cigarettes but not the capsule itself. Capsules for self-administration and menthol cards are available in fruity, sweet and mint-like flavours and in different colourings. So far, no studies are available on the composition of these flavour capsules or the compounds present in flavouring cards.


**Figure 4-2: Flavour capsules (A) and menthol cards (B).**

# 5 Conclusions and Outlook

The present work elaborates on the establishment of reliable analytical methods to address the implementation of Articles 6 and 7 of the EU Tobacco Products Directive (TPD) 2014/40/EU. Fife years after the Directive became applicable in May 20, 2016, the first Application Report was released and revealed that sufficient data satisfying these Articles is still absent. Up to date very little experimental work has been focussed on the assessment of tobacco additives under smoking conditions. Only a series of three studies, conducted by the tobacco industry, reported on the evaluation of so-called priority additives (Simms et al., 2019; Stabbert et al., 2019; McEvan et al., 2019).

The suggestion to perform pyrolysis experiments was rejected by the industry with the argument that such conditions would not provide data on the real chemical composition in tobacco mainstream smoke (Stabbert et al., 2019). Yet, information on degradation products of tobacco additives is still necessary to fulfil the requirements of the TPD. The oxidative py-GC/MS method established in this thesis work can serve as a reliable tool for the screening of such degradation products. However, in terms of the assessment of hazardous compounds that might be formed under smoking conditions from tobacco additives, quantitative methods are needed as well.

The option to use the Common Entry Gate (EU-CEG) to screen the provided industry data of the tobacco additives for marker substances of characterising flavours, was neglected by the European Commission. The possibility to chemically define key substances that confer a flavouring note to the product has been proven by the present work. The screening of the EU-CEG database for such combinations in the manufactured cigarettes would be an effective tool for the surveillance.

The menthol studies conducted in this thesis demonstrate that menthol contents beyond the sensory threshold are sufficient to trigger TRPM8 receptor activation. This experimental outcome already affected the regulation of menthol in tobacco products in our country. In Germany any addition of menthol in cigarettes and RYO has been prohibited by the implementation of the TabakerzV. In most other Member States of the EU the mentholating of cigarettes is regulated only based on its possibility to create a characterising flavour. This, in turn, results in the assumption that menthol levels below the sensory threshold but sufficient to activate the TRPM8 receptor are still allowed in most other Member States. According to our studies, such menthol amounts yet would contribute to ease smoke inhalation and thus to increase the addictive potential of the product.

Eight years after implementation of the TPD, the collected analytical and toxicological data sets are still insufficient to enable a robust and reliable risk assessment of tobacco additives. This situation sheds light on the complexity of the issue of smoke chemistry.

Ultimately, all efforts to fulfil the requirements of the TPD seem to be no more than waste of time and resources, if it becomes possible to simply bypass the legal ban of characterising flavours by the use of alternative items such as flavouring capsules or menthol cards. It finally has to be emphasised that such new technological product developments urgently need to be addressed in the Application Report of the European Commission.

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