Determination of nicotine delivery and emissions of hazardous substances from electronic cigarettes and heated tobacco products

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Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel

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Zusammenfassung

Zigarettenrauch enthält neben dem Suchtstoff Nikotin eine Vielzahl von Schadstoffen, viele davon sind bekannte Kanzerogene. Auf Grund der hohen Raucherquote stellt Zigarettenrauchen daher ein sehr großes, aber dennoch vermeidbares gesundheitliches Risiko dar. Obwohl ein kompletter Rauchstopp die effektivste Methode ist, um Tabak-assoziierte gesundheitliche Schäden zu verringern, fällt es vielen Rauchern schwer ihre Tabakabhängigkeit zu überwinden. Alternative Nikotinabgabesysteme geben Nikotin über andere Mechanismen im Vergleich zu Tabakzigaretten ab. Dies führt mutmaßlich zu einer geringeren Exposition gegenüber schädlichen Pyrolyseprodukten. E-Zigaretten nutzen elektrisch generierte Wärmeenergie, um eine Mischung aus Feuchthaltemitteln und anderen Inhaltsstoffen, insbesondere Nikotin, in eine inhalierbare Form zu überführen. Bei Tabakerhitzern wird auf ähnliche Weise der enthaltene Tabak erhitzt, um Nikotin ins Aerosol abzugeben. Beide Produktgruppen sind in sich sehr heterogen. Es kommen unterschiedliche Verfahren zum Einsatz, um bei Tabakerhitzern die Wärmeenergie auf den Tabak zu übertragen. Auch das Design von E-Zigaretten unterliegt regelmäßigen Generationswechseln. Beispielsweise gab es in den letzten Jahren einen Trend zu E-Zigaretten, die sich durch ein simples Design und eine einfache Benutzung auszeichnen. Die Nutzung von Nikotinsalzen erlaubt zudem den Einsatz von hohen Nikotinkonzentrationen (bis hin zu fast 60 mg/mL). Dieser Trend führte zu einem starken Anstieg an jugendlichen E-Zigaretten-Nutzern in den USA und hatte damit schwerwiegende Folgen für die öffentliche Gesundheit. Die europäische Tabakproduktrichtlinie (engl. tobacco product directive, TPD) begrenzt hingegen den Nikotingehalt in Liquids für E-Zigaretten auf ein Maximum von 20 mg/mL. Wenn neue oder modifizierte alternative Nikotinabgabesysteme auf dem Markt eingeführt werden, ist daher anfänglich wenig Wissen vorhanden, um das Abhängigkeitspotential und die potenzielle Schädlichkeit der Produkte abzuschätzen.

Eine wissenschaftlich fundierte Risikobewertung der Produkte benötigt Daten zur Abgabe von Nikotin und weiteren schädlichen Substanzen an den Konsumenten. Während eine Reihe standardisierter Methoden für die Analytik von Zigarettenrauch existiert, bedarf die chemische Charakterisierung der neuen Produkte zunächst einer Anpassung oder Neuentwicklung der Methoden. Daher wurden neben dem Nikotingehalt auch die Hauptkanzerogene im Zigarettenrauch, volatile organische Verbindungen und Carbonylverbindungen, in den Hauptstromemissionen zweier Tabakerhitzer untersucht. Standardmethoden für die Untersuchung von Zigarettenrauch wurden für diesen Zweck angepasst und auf ihre Eignung überprüft. Zusätzlich wurde die thermische Zersetzung eines neuartigen Filtermaterials, welches bei einem der Tabakerhitzer zum Einsatz kommt, simuliert und mögliche Zersetzungsprodukte von toxikologischer Relevanz wurden identifiziert. Der Gehalt an Nikotin und an Carbonylverbindungen in den Hauptstromemissionen einer E-Zigarette, die kürzlich auf dem europäischen Markt eingeführt wurde, wurde bestimmt. Eine lineare Abdampfmaschine wurde für die

Untersuchung der Tabakerhitzer und der E-Zigarette genutzt. Diese Maschine ist dafür ausgelegt, um mit einem standardisierten Verfahren Aerosol aus E-Zigaretten zu erzeugen. Weiterhin wurde die Nikotinaufnahme nach tatsächlicher Nutzung der untersuchten E-Zigarette in einer Humanstudie mit erfahrenen E-Zigaretten-Nutzern untersucht. Studienteilnehmer wurden instruiert, ihr Testprodukt nach einem vorgegebenen Zugschema zu nutzen. Zu diesem Zweck wurde vorher eine Bestimmungsmethode für Nikotin und dessen Hauptmetabolite aus Humanplasma entwickelt und nach bioanalytischen Leitlinien validiert.

Die europäische Version der untersuchten E-Zigarette zeigte im Vergleich zur amerikanischen Variante eine reduzierte Nikotinabgabe als Folge der etwa auf ein Drittel reduzierten Nikotinkonzentration im Liquid. Eine technische Modifikation des Dochtmaterials seitens des Herstellers führte zu einer erhöhten Nikotinabgabe bei maschineller Aerosolerzeugung. Dennoch waren die erreichten Nikotinkonzentrationen im Blut humaner Probanden nach Nutzung der modifizierten E-Zigarette wesentlich geringer im Vergleich zum Konsum einer Tabakzigarette. Auch die Nikotinanflutung in den ersten Minuten der Produktnutzung war deutlich langsamer für beide europäische Varianten (vor und nach der Produktmodifikation) im Vergleich zur Nutzung einer konventionellen Tabakzigarette. Es zeigte sich, dass die technische Modifikation nicht ausreichte, um den reduzierten Nikotingehalt im Liquid bei tatsächlicher Nutzung des Produktes vollständig zu kompensieren.

Die untersuchten Kanzerogene wurden in den Emissionen beider Tabakerhitzer im Vergleich zum Zigarettenrauch in deutlich geringeren Konzentrationen nachgewiesen. Volatile organische Verbindungen, insbesondere 1,3-Butadien, welches einen wesentlichen Anteil an der Kanzerogenität von Tabakrauch beiträgt, waren um mehr als 96% reduziert. Die Reduktion der Carbonylverbindungen in den Emissionen beider Produkte war weniger deutlich. Auch die Nikotinabgabe pro Konsumeinheit war geringer als üblicherweise im Zigarettenrauch. Ein Tabakerhitzer lag in einem ähnlichen Bereich wie Zigaretten mit geringer Nikotinabgabe, das andere Produkt gab im Vergleich dazu nur ein Drittel der Nikotinmenge ab. Existierende Analysemethoden für Zigarettenrauch konnten für die Charakterisierung der Tabakerhitzer angepasst werden, mit Ausnahme der Methode zur Wasserbestimmung. Der hohe Wassergehalt in den Emissionen der Tabakerhitzer begünstigen dessen Unterschätzung. Der Wassergehalt wird zur Berechnung des Teergehaltes in Zigarettenrauch benötigt, welcher durch die TPD begrenzt ist. Im E-Zigaretten-Aerosol wurden ebenfalls Carbonylverbindungen gefunden, allerdings nahe der Bestimmungsgrenze.

Raucher, die komplett auf Tabakerhitzer oder E-Zigaretten umsteigen, reduzieren wahrscheinlich ihre Exposition gegenüber krebserregenden Substanzen, wenn sie ansonsten weitergeraucht hätten. Diese Schadstoffreduktion könnte durch kompensatorische Produktnutzung auf Grund einer geringeren Nikotinabgabe vermindert werden. Die Kehrseite einer hohen Nikotinabgabe, insbesondere mit einer

schnellen Nikotinanflutung in der akuten Phase, ist eine potenzielle Suchtauslösung. Die europäische Version der untersuchten E-Zigaretten führte zu einer geringen Nikotinabgabe bei Produktnutzung mit einer langsamen Anflutung. Dies ist eine Konsequenz der limitierten Nikotinkonzentration im Liquid in Kombination mit den vom Nutzer nicht modifizierbaren Geräteeinstellungen. Aus den Daten lässt sich auf ein geringeres Suchtpotential dieser Produktvariante schließen. Gesetzliche Regulierungen von alternativen Nikotinabgabesystemen können die Exposition der Konsumenten gegenüber schädlichen Substanzen und das Abhängigkeitspotential der Produkte beeinflussen. Sowohl die Regulierung als auch die wissenschaftlichen Grundlagen dafür müssen kontinuierlich erweitert und angepasst werden, um mit den rapiden Entwicklungen auf dem Markt schritthalten zu können, z.B. bei der Einführung neuer Produktgruppen und neuartiger Konstruktionsmerkmale.

Abstract

In addition to the reinforcing compound nicotine, cigarette smoke contains a vast amount of hazardous chemicals, many of them known carcinogens. In combination with high smoking prevalence, cigarette smoking poses a huge yet avoidable health risk. Although smoking cessation is the most effective way to reduce smoking-associated harm, tobacco dependence is difficult to overcome. Alternative nicotine delivery systems (ANDS) release nicotine using different mechanisms than tobacco cigarettes, supposedly associated with a lower exposure to hazardous pyrolysis products. E-cigarettes use electrically generated heat to aerosolize a mixture of humectants and other ingredients to bring nicotine into an inhalable form. Similarly, heated tobacco products (HTPs) apply heat to aerosolize nicotine from tobacco. Both product groups are heterogeneous; HTPs employ different heating mechanisms and the design of e-cigarettes undergoes a constant change in generations. In recent years, e-cigarettes became simpler in use. Application of nicotine salts enabled the delivery of high nicotine concentrations (up to almost 60 mg/mL) that has led to a rise in adolescent users of e-cigarettes in the US and consequently to serious public health concerns. In Europe, the nicotine content in liquids of e-cigarettes is limited to a maximum of 20 mg/mL by the Tobacco Product Directive (TPD). Consequently, when such new or modified products enter the market, there is initially little knowledge regarding their potential addictiveness and harm.

Science-based risk assessment of these products requires data on the delivery of nicotine and hazardous compounds to the consumer. While plenty of standardized methods already exist for the analysis of cigarette smoke, chemical characterization of novel products requires their adaption or development of new methods. Thus, nicotine and the main contributors to carcinogenicity of tobacco smoke, volatile organic compounds (VOCs) and carbonyl compounds, were determined in mainstream emissions of two different HTPs. Standard methods for cigarette smoke were adapted for this purpose testing their feasibility for the characterization of the product group. Thermodegradation products of a novel filter material used in one HTP were followed up to identify potentially new compounds with toxicological relevance. Content of nicotine and carbonyl compounds was assessed in mainstream emissions of an e-cigarette brand that was recently introduced on the European market. A linear vaping machine designed for standardized aerosol generation of e-cigarettes was used for emission generation of HTPs and e-cigarettes. Further, nicotine delivery by actual consumption of the studied e-cigarette was followed up in a clinical study with experienced e-cigarette users. Participants were instructed to use their study products according to a pre-directed puffing protocol. For this purpose, a quantitation method for nicotine and its main metabolites from human plasma was developed and validated in accordance with bioanalytical guidelines.

Nicotine delivery by the European version of the investigated e-cigarette was reduced compared with the US version as a consequence of threefold lower liquid nicotine content. A technical modification of the wick material used has resulted in an increased nicotine delivery in the vaping machine study. Nevertheless, nicotine concentrations in the blood of human participants that were achieved upon consumption of the e-cigarette were significantly lower in comparison to the nicotine concentrations in blood after smoking of a conventional cigarette. Further, the increased rate of nicotine concentration in the blood in the first minutes of consumption was low for the initial and the modified European variant compared with consumption of a conventional cigarette. At stage of consumption, the technical modification of the wick material was not sufficient to compensate for the lower liquid nicotine content.

The analyzed carcinogens were reduced in the emissions of both HTPs in comparison to cigarette smoke. VOCs, especially the major contributor to cigarette smoke carcinogenicity 1,3-butadiene, were reduced by more than 96%. A reduction of carbonyl compounds by both products was apparent but less pronounced. Nicotine delivery per consumable unit was lower than usually measured for cigarettes. One device delivered a similar amount of nicotine as low yield cigarettes, while the nicotine delivery of the second device was approximately one third compared with the first device. Existing analytical methods for determination of cigarette smoke could be adapted for their application for HTPs, except for determination of water content. High water contents in product aerosols, such as seen for HTPs, are underestimated with water determination methods standardized for cigarette smoke. The amount of water in tobacco product emissions is used to calculate the value “tar” which has an upper limit in cigarette smoke set by TPD. Carbonyl compounds were detected in e-cigarette aerosol as well, yet close to their lower limits of quantitation.

Smokers who would otherwise continue smoking are likely to reduce their exposure to carcinogenic substances by completely switching to HTPs and e-cigarettes. This reduction could be lessened by possible compensatory effects caused by reduced nicotine delivery. The other side of a high nicotine delivery, especially a quick elevation of blood nicotine levels in the acute phase, is a potential induction of dependence. The limited nicotine content in the liquid of the European variant of the e-cigarette in combination with its fixed settings for vaporization result in low nicotine concentrations in blood with a slow rise. This suggests a lower potential of the product to induce addiction as seen with e-cigarettes with high nicotine concentrations in the liquid. Regulation of ANDS can have an impact on the consumer’s exposure to harmful substances and the product’s potential addictiveness. Regulation and the supporting scientific basis have to be under constant progression to keep pace with novelties on the market such as new product groups or unconventional design features.

Abbreviations

ANDS	Alternative Nicotine Delivery Systems
BDL	Below detection limit
BMD	Benchmark dose
BoE	Biomarker of Exposure
Carc.	Carcinogen
CCE	Change in cumulative exposure
CLP	Classification, Labelling and Packaging (Regulation (EC) No 1272/2008)
C_{max}	Maximum plasma concentration
CORESTA	Cooperation Centre for Scientific Research Relative to Tobacco
CPF	Cancer potency factor
CRM	CORESTA Recommended Method
CYP	Cytochrome P450
DAD	Diode array detector
DIN	Deutsches Institut für Normung
ECHA	European Chemicals Agency
ESI	Electrospray ionization
EU	European Union
FDA	Food and Drug Administration
FID	Flame ionization detection
FTND, FTCD	Fagerström test for nicotine dependence, Fagerström test for cigarette dependence
GC	Gas chromatography
HCI	Health Canada Intense
HnB	Heat not Burn
HPHC	Harmful and Potentially Harmful Constituents
HS	Headspace
HTP	Heated tobacco products
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization
ISTD	Internal Standard
LC	Liquid chromatography
MHBMA	Monohydroxy-3-butenyl mercapturic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

nAChR	Nicotinic acetylcholine receptor
NFDPM	Nicotine-free dried particulate matter
NMR	Nicotine metabolic ratio
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N*'-nitrosonornicotine
NRT	Nicotine replacement therapy
PAH	Polycyclic aromatic hydrocarbons
PG	Propylene glycol
PLA	Polylactic acid
Pyr	Pyrolysis
QSU	Questionnaire on smoking urges
REL	Reference exposure level
rH	Relative Humidity
RPF	Relative potency factor
SPME	Solid Phase Microextraction
TCD	Thermal conductivity detector
t_{max}	Time point of maximum plasma concentration
TNCO	Tar, nicotine, and carbon monoxide
TobLabNet	Tobacco Laboratory Network
TobReg	Study Group on Tobacco Product Regulation
TPD	Tobacco Product Directive (Directive 2014/40/EU)
TPM	Total particulate matter
TSNA	Tobacco-specific *N*-nitrosamines
VG	Glycerol, vegetable glycerol
VOC	Volatile organic compound
WHO	World Health Organization

Abbreviations exclusively used within the publications included in this thesis are not specified in this list.

1. Introduction

Tobacco and nicotine delivery products have been under intensive development in recent years. While extensive research has already been performed on conventional tobacco cigarettes, wide knowledge gaps exist for emerging nicotine delivery products. The first key points to be clarified for their understanding and risk assessment are the products' nicotine delivery and their emissions and/or constituents with toxicological relevance.

1.1. Tobacco smoking

Tobacco smoking is a major risk factor for chronic noncommunicable diseases, such as different types of cancer, respiratory and cardiovascular diseases, diabetes, fractures, arthritis, blindness, peptic ulcer disease, and others [1-9]. Worldwide, 1.14 billion people were smokers in 2019 with an age-standardized prevalence of 32.7% for men and 6.62% for women [10]. Prevalence in the European Union was higher with 30% for men, 22% for women, 26% current smokers in total and 20% former smokers in 2017 [11]. Numbers from 2014/2015 on smoking behavior in Germany were similar to the European numbers: 23.8% total current smokers (daily or at least regularly), with 27.0% for men and 20.8% for women, and 30.7% former smokers [12]. Although smoking prevalence is decreasing in Germany and worldwide, smoking is still a relevant factor for premature death [1, 2]. Of the 954,800 deaths in Germany in 2018, approximately 127,000 (13.3%) were attributable to tobacco smoking [1]. Among men, 17.7% of deaths were attributable to tobacco [1]. Worldwide, 11.5% of deaths (6.4 million) were attributable to tobacco smoking as of 2015 [2]. According to the latest Global Burden of Disease report for 2019, deaths attributable to tobacco smoking had increased to 13.6% (i.e., 7.69 million) [10]. Among males, at 20.2% smoking was the leading risk factor for death [10]. Further, 7.89% of disability-adjusted life-years were attributable to tobacco smoking [10]. These numbers underline the importance of tobacco control policies aiming at a further reduction of smoking prevalence but also tobacco-induced harm. Therefore, although the link between tobacco smoking and increased mortality has been made decades ago [3], science-based risk assessment for tobacco and nicotine products, especially alternative nicotine delivery systems, is still necessary.

1.1.1. Smoke chemistry

Cigarette smoke is a complex mixture of more than 5000 chemicals [13], some of them of high toxicological concern. Hoffmann and coworkers have identified and published several compounds in cigarette smoke that are biologically or toxicologically active [14, 15], commonly referred to as "Hoffmann analytes". Talhout *et al.* have suggested a list of 98 hazardous smoke components for regulatory purposes [16]. United States Food and Drug Administration (FDA) has established a list of 93 harmful and potentially harmful constituents (HPHC) [17], and the Study Group on Tobacco Product

Regulation (TobReg) of the World Health Organization (WHO) listed 39 toxicologically relevant compounds that should be monitored with priority [18]. These lists comprise different compound groups, especially aldehydes and other volatile organic compounds (VOCs), polycyclic aromatic hydrocarbons (PAHs), aromatic amines, tobacco-specific *N*-nitrosamines (TSNAs), metals, and inorganic compounds. Nine of them (acetaldehyde, acrolein, 1,3-butadiene, benzene, benzo[*a*]pyrene, carbon monoxide, formaldehyde, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and *N*'-nitroso-nornicotine (NNN)) were considered by WHO TobReg to be the most hazardous, representing differences in compound groups and formation pathways. Therefore, they were proposed for a mandatory reduction in cigarette smoke [19]. Fowles and Dybing have ranked smoke constituents based on their emission levels and cancer potency factors (CPFs) or reference exposure levels (RELs). They concluded that aldehydes and other small organic compounds, such as 1,3-butadiene, acrylonitrile, and acetaldehyde, were the main contributors to carcinogenicity of cigarette smoke [20].

Formation of these compounds is complex and depending on many parameters like temperature, oxygen level, and pH [21]. A burning cigarette can be divided into different zones where different processes take place under specific conditions. The "combustion zone" at the tip can reach temperatures between 700 and 950°C [22]. Oxygen reacts with tobacco compounds, generating carbon monoxide, carbon dioxide, hydrogen cyanide, water, and the heat that sustains the burning process [21, 22]. Further inside the cigarette, the temperatures are lower, between 200 and 600°C [22]. In this "pyrolysis and distillation zone", most smoke constituents are formed in endothermic reactions [22]. The saturated vapor is then cooled to below 350°C by ambient air that is drawn in at the paper burn line [22]. In this "condensation zone", droplets are formed [22]. Carbonyl compounds like acrolein, acetaldehyde, formaldehyde, and aromatic amines are formed at lower temperatures below 500°C, while benzo[*a*]pyrene, benzene, 1,3-butadiene, and acrylonitrile are formed at higher temperatures of 500 to 800°C [21]. TSNAs are formed from nicotine and other alkaloids during curing and processing of tobacco leaves and are already present in unburnt tobacco [23]. Similarly, metals are already present in unburnt tobacco and transfer into mainstream and sidestream smoke [24]. Tobacco plants take up metals from the soil and even enrich cadmium to much higher concentrations compared with food [24]. Taken together, active and passive tobacco smoking leads to relevant exposure to several substances of high toxicological concern, posing a substantial yet avoidable health risk.

1.1.2. Simulation with smoking machines

To analyze and quantify smoke constituents, cigarette smoke can be generated automatically by utilization of smoking machines. On one side of a smoking machine is the burning cigarette, on the other side is a piston pump that draws puffs in a standardized manner. In between, the analyst can trap the smoke constituents with different methods. Particulate matter is usually collected on Cambridge glass

fiber filters (Cambridge filter medium No. CM-113, developed by Cambridge Filter Corp., Syracuse, NY, USA), including analytes such as nicotine that can be extracted and analyzed [25, 26]. Gas vapor phase constituents can be collected, amongst other methods, with impingers containing an appropriate solvent [27-29], on cartridges [30], or in air-tight bags [31]. **Figure 1** gives a schematic overview of one channel of a linear smoking machine where the mainstream smoke of individual cigarettes can be collected separately. These multi-channel machines usually have one pump that draws puffs from the different test cigarettes successively. The air flow through the channels each equipped with filters and/or impingers is directed by valves. In other types of smoking machine, the emissions from the tested cigarettes might be combined in the same airflow with one filter and/or set of impingers. Rotary smoking machines are built with a carousel that moves the cigarettes successively to the single smoking port where the puff is drawn.

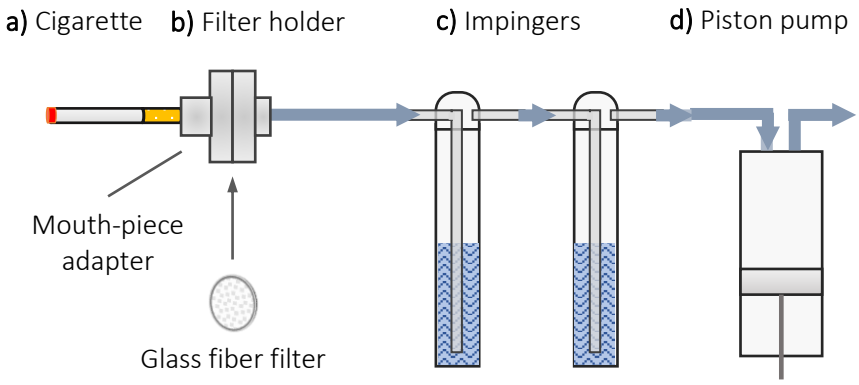

Figure 1. Schematic display of a linear smoking machine. **a)** The test item, a cigarette or other product, is attached to a mouth-piece adapter that can be a mounted to **b)** a filter holder containing a filter to collect total particulate matter. **c)** Impingers to trap gas phase constituents from filtered or unfiltered aerosol can be installed before the **d)** piston pump that creates the puff under defined conditions.

The pumps are programmed to puff with defined regimens that were designed to mimic actual smoking. International Organization for Standardization (ISO) has defined a smoking regimen in its standard ISO 3308. Puffs are drawn every 60 s for 2 s with a bell-shaped puffing profile, puff volume is 35 mL [32]. This regimen was first proposed in 1936 by Bradford *et al.* [33] and is now often referred to as the "ISO method". The European Tobacco Product Directive (TPD, 2014/40/EU) specifies this method for emission testing (Art. 4) [34]. Health Canada has defined a smoking regimen that resembles a more intense smoking behavior with a higher temperature generation resulting in the Health Canada Intense (HCI) standard. Puffs are drawn for 2 s with a bell-shaped puffing profile, but with a frequency of 30 s and a puff volume of 55 mL [35]. Another major difference between both regimens is that HCI requires the cigarette filter paper to be tape sealed to cover ventilation holes. Due to the blocked filter ventilation holes and the accelerated burning process, toxicant yields are higher when the HCI smoking regimen is used compared with the ISO method [36]. WHO Tobacco Laboratory Network (TobLabNet) has introduced an intense puffing standard (WHO intense) with the same parameters as HCI [37]. There

exist further smoking regimens that are not used as widely as ISO and Health Canada/WHO intense and were not used in this thesis. Prior to smoke generation, cigarettes are conditioned with the same standard climatization as the atmosphere for testing, inside the smoking machine and in the whole room (22 °C and 60% relative humidity, rH), according to ISO 3402 [38].

It should be noted that these machine puffing regimens cannot be used to generate realistic exposure data as they do not mimic human smoking adequately [39, 40]. However, derived quantities of smoke constituents can be useful to compare different products with each other enabling a regulation of maximum emission levels. For example, TPD limits emission levels of tar, nicotine, and carbon monoxide (TNCO) from cigarettes that are determined using the ISO method [34]. WHO TobReg advises to use WHO intense regimen to regulate priority toxicants in mainstream smoke [18].

1.1.3. Nicotine and tobacco dependence

Tobacco smoking is highly addictive, leading to strong psychological dependence. In a scoring system for drugs of potential misuse, only heroin and cocaine scored higher for dependence [41]. A dependence syndrome is defined by the WHO as a combination of physiological, cognitive, and behavioral components, often associated with an overpowering desire to consume the addictive substance [42]. In a representative survey conducted in Germany in 2019, only one in five smokers reported to have undertaken at least one quit attempt in the last year [43]. Of those smokers and recent ex-smokers, merely 13% had used an evidence-based method to support their last quit attempt [43]. E-cigarettes with and without nicotine, that are not listed among evidence-based cessation aids in current German guidelines [44], were chosen by 10% for cessation [43]. The authors of a literature review concluded that only 3-5 % of smokers without treatment are successful in their quitting attempt for longer than twelve months [45]. Relapse to smoking mostly occurred within the first eight days [45]. Available treatments for tobacco dependence include behavioral interventions (e.g., counselling, self-help materials, group interventions), pharmacotherapy (e.g., varenicline, a nicotine receptor partial agonist, or bupropion, a non-tricyclic antidepressant) and nicotine replacement therapy (NRT), e.g., nicotine patches or nicotine gums, and combinations of different forms of treatment [44, 46-48].

Pharmacokinetics and -dynamics of nicotine from cigarettes

An activation of the reward link via dopamine release in the mesolimbic system can result in positive reinforcement [49]. Inhaled nicotine is quickly absorbed in the lungs and transported to the brain within 10-20 s [50]. Arterial nicotine concentration after consumption of one cigarette typically ranges between 20 to 60 ng/mL and was even reported with up to 90 ng/mL [50, 51]. The rapid “rush” of a drug in the brain has been linked to pleasure sensation and development of addiction [52]. This association is discussed for nicotine dependence as well [51]. Inside the brain, nicotine binds to nicotinic

acetylcholine receptors (nAChR) in the mesolimbic ventral tegmental area where it subsequently depolarizes dopaminergic neurons, releasing dopamine in the shell of the nucleus accumbens [53, 54]. Desensitization of nAChR subtype $\alpha_4\beta_2$ is considered to be responsible for nicotine addiction and withdrawal symptoms [53, 54]. In addition, other nAChR subtypes contribute to addiction [55].

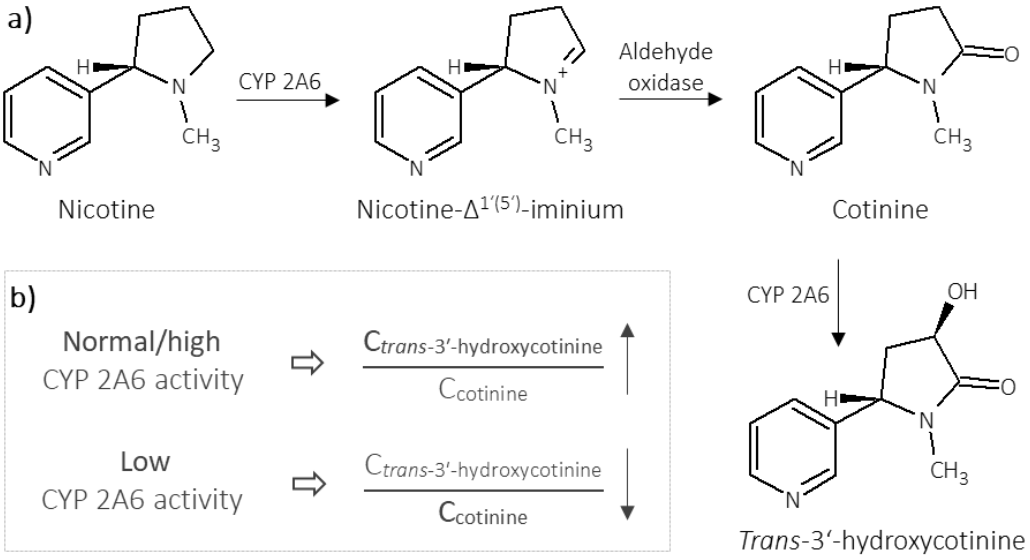

Figure 2. a) Main route of nicotine metabolism resulting in metabolites cotinine and *trans*-3'-hydroxycotinine mediated by CYP 2A6 (based on Hukkanen *et al.* [50]). **b)** Relationship between CYP 2A6 activity and nicotine metabolic ratio (ratio of concentrations of *trans*-3'-hydroxycotinine and cotinine).

Concentrations of nicotine in venous plasma after consumption of one cigarette were reported between 5 and 30 ng/mL [50]. Nicotine has a distribution half-life of 9 min, and a terminal half-life of about 130 min [56]. Nicotine is mainly metabolized to cotinine and in the following to *trans*-3'-hydroxycotinine, both via the cytochrome P450 (CYP) isoform 2A6 as summarized in **Figure 2a** [50, 57-60]. Polymorphisms of CYP 2A6 can lead to a reduction of activity [61]. High/normal enzyme activity has been positively correlated with severity of nicotine dependence and withdrawal symptoms [62]. Since transformation from nicotine to cotinine and from cotinine to *trans*-3'-hydroxycotinine are mediated via CYP2A6, the blood concentration ratio of both metabolites, referred to as "nicotine metabolic ratio" (NMR), can be used as a biomarker for CYP2A6 activity [63, 64]. NMR is calculated by dividing the concentration of *trans*-3'-hydroxycotinine by the concentration of cotinine as shown in **Figure 2b**. The faster the metabolism from cotinine to *trans*-3'-hydroxycotinine, the higher the value for NMR [63, 64]. In clinical trials, NMR had an influence on effectiveness or on side effects of smoking cessation treatments [65-67]. Cut-off NMR values to distinguish between slow and normal/rapid metabolizers have been used with 0.26 or 0.31 [65, 67].

Tobacco dependence

In addition to desensitization of $\alpha_4\beta_2$ nAChR, other factors play important roles in tobacco dependence. Cigarettes are consumed by dependent smokers for positive effects (positive reinforcement) and for avoidance or termination of withdrawal symptoms or cravings (negative reinforcement) [68, 69]. Withdrawal symptoms and cravings can be produced by the absence of nicotine or by a conditioned response of the body to a drug-associated stimulus (e.g., sensory cues) [69]. One such sensory cue is a nicotine-induced sensation of harshness in the throat, referred to as "throat hit" [70]. The throat hit is an important cue also in the context of e-cigarette consumption [71]. Aspects of social learning, for example outcome expectancy, abstinence self-efficacy, coping, and automaticity, contribute to dependence [72]. Further, other substances in tobacco smoke are discussed for their contribution to addiction. Acetaldehyde reacts with biogenic amines to inhibitors of monoamino oxidase, an enzyme that metabolizes dopamine and other neurotransmitters, potentially enhancing reinforcing effects of nicotine [73].

Questionnaires for assessment of craving like the "Questionnaire on Smoking Urges" (QSU) and the validated German version QSU-G inquire items for positive and for negative reinforcement [74, 75]. Tobacco dependence can be assessed with questionnaires such as the "Fagerström test for nicotine dependence" (FTND), also termed "Fagerström test for cigarette dependence" (FTCD) [76, 77]. Test items comprise the time to the first cigarette in the morning, difficulty to refrain from forbidden cigarettes, which cigarette would be the worst to give up (first cigarette in the morning vs. other), cigarettes smoked per day, whether subjects smoke more frequently in the morning than during the rest of the day, and whether they smoke when they are ill [76].

1.1.4. Alternative Nicotine Delivery Systems (ANDS)

As alternatives for combustible cigarettes, various products have been designed for administration of nicotine. NRT aims for a delivery of nicotine that suppresses the urge to smoke but does not induce addictive behavior [78, 79]. The products are usually in pharmaceutical quality and registered as medicinal products to aid smoking cessation. Nicotine is administered in different forms, e.g., as patches, gums, inhalers, or sprays, resulting in flatter nicotine plasma curves than cigarette smoking [80, 81]. Importantly, these products do not lead to a nicotine "rush" in the brain [80, 81]. This is considered to be responsible for their absence of induction of nicotine dependence in naïve users [78, 79].

Other alternative products have been developed and are mainly sold as consumer products. They administer nicotine orally, like snus [82] or nicotine pouches [83], or via inhalation, like electronic cigarettes or heated tobacco products (HTP). The latter two product types have been studied in this work and are introduced in more detail under **1.2. Electronic cigarettes** and **1.3. Heated tobacco**

products. These products are not registered as medicinal cessation aids and are often part of the extended portfolio of cigarette manufacturers [84]. Nevertheless, such alternative nicotine delivery systems (ANDS) are preferred by some addicted smokers to support their smoking cessation [43].

Further, they can be part of harm reduction strategies aiming at a lowering of health risks if a complete exclusion of risks cannot be achieved under the given circumstances [85-88]. Harm reduction strategies have been successfully employed for prevention of bloodborne viral infections in users of illicit drugs, for example by provision of needle and syringe exchange, drug consumption rooms, and substitution therapy [89]. Key criteria for harm reduction are the focus on the drug user's individual health risk and the acceptance of the user's choice to continue drug use [90]. Thus, they are oriented on short term goals, although a complete absence of drug use could remain an ultimate goal [90]. In terms of tobacco addiction, harm reduction strategies can aim towards a switch to nicotine delivery products with reduced toxicant exposure in smokers that are unwilling or unable to quit smoking. While low tar cigarettes have been shown to be ineffective in reducing health risks [88, 91-93], nicotine can be administered in the form of NRT or potentially in the form of ANDS as a less harmful alternative [85-88].

However, ANDS are highly controversial as their risks, their potential addictiveness, and their impact on population health is still not fully understood [88]. In addition, their safety and efficacy are not controlled unlike for medicinal products [88].

1.2. Electronic cigarettes

E-cigarettes produce an inhalable aerosol usually by applying heat to a so-called liquid (also e-liquid). The liquid consists of a "basis", mostly a mixture of glycerol (also referred to as vegetable glycerol, VG) and propylene glycol (PG), with optional additives like nicotine and aroma compounds. A wick, often made of cotton, transports the liquid to an electrical heating coil. The generated heat vaporizes the liquid that is then inhaled by the consumer. Following this general principle, different product types, sometimes referred to as different generations, have been designed. Schematic presentations are shown in Figure 3.

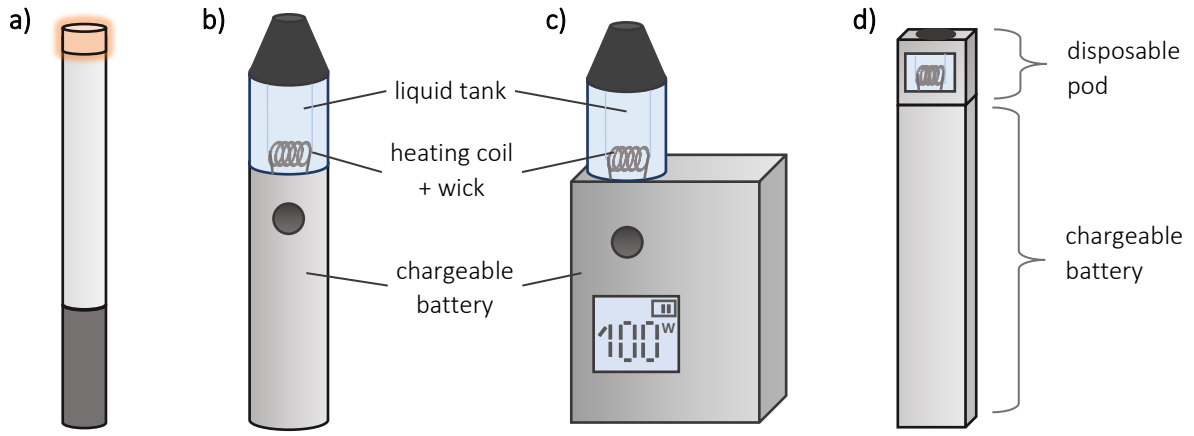

Figure 3. Different types of electronic cigarettes. **a)** Disposable "cigalikes", **b)** tank models, also referred to as "mods", consisting of refillable liquid tank, exchangeable heating coil and chargeable battery, **c)** high power devices, also referred to as "sub ohm" electronic cigarettes, with a high-capacity battery and a low resistance heating coil, and **d)** pod systems with a disposable pod, containing prefilled liquid and coil, and chargeable battery.

The appearance of the first e-cigarettes was aimed to resemble combustible cigarettes (Figure 3a). These "cigalikes" (from "cigarette alike") were single-use products with a low vapor generation [94-96]. Later generation e-cigarettes had a modular composition with battery, liquid tank, heating coil, and wick (Figure 3b) [94-96]. Coil and wick of these "mods" can be replaced, the tank can be refilled with a liquid of choice and the battery can be recharged or replaced [94-96]. Technical modification of this product type has advanced in so-called "sub ohm" devices (Figure 3c). The combination of a battery with high capacity and a heating coil with low resistance of below 1 Ω enables high power vaporization up to 300 W [97]. The large volume of generated vapor, approximately half a liter, is directly inhaled into the lung, similarly to emissions of waterpipes [98, 99]. The recently emerged trend of pod e-cigarettes (Figure 3d) went in the opposite direction: the products are simple to use, do not require knowledge of electrotechnics, and usually produce a lower amount of vapor. Another characteristic of this product type is that it may contain high nicotine concentrations in the liquid of 50 mg/mL and more [96, 100]. While unprotonated "free-base" nicotine is responsible for the throat hit that serves as a sensory cue,

such high concentrations of free-base nicotine would lead to an adverse sensation that prevents a deep inhalation [70, 71, 101]. The addition of a weak organic acid such as benzoic or salicylic acid lowers the pH value and increases the grade of protonation of nicotine [102, 103]. Thus, the amount of free-base nicotine and the adverse sensation is reduced, and an inhalation of high nicotine concentrations is possible [101, 103].

1.2.1. Puffing behavior and nicotine delivery

Delivery of nicotine into the blood after e-cigarette use depends on many different factors, such as user experience and puffing behavior, device type and design, power output, liquid composition, flavors, and nicotine concentration [104-108]. The most important factor, the puffing behavior, is likewise influenced by the mentioned factors. In a clinical study, St. Helen *et al.* have observed that vaping patterns differ from cigarette smoking with shorter clusters of puffs and an intermittent nicotine dosing [109]. Twelve percent of puffs were unclustered and 43% of puffs were taken in short clusters of 2-5 puffs [109]. Experienced e-cigarette users were shown to take puffs of 4 s, longer in comparison to cigarette smokers and unexperienced e-cigarette users with about 2 s [104, 109, 110]. High nicotine concentrations in the liquids (36 mg/mL) have led to a significantly shorter puff duration than consumption of nicotine-free liquids [111]. Strawberry flavor had an increasing effect on puff duration compared to tobacco flavored liquids [108]. However, when using their preferred liquid brand, puffs were even longer [108]. The authors of this study have discussed the pH value of the liquid to be a potential influencing factor for puffing behavior [108]. As discussed above, high amounts of free-base nicotine can lead to adverse sensations. A decrease of the pH value and subsequently of the amount of free-base nicotine by flavor components can increase palatability [106, 108, 112].

Consumption of first-generation e-cigarettes resulted in a low nicotine delivery, whereas use of later-generation products can lead to higher blood nicotine concentrations [113, 114]. Nicotine delivery of newer e-cigarettes, especially but not exclusively nicotine-salt containing pod e-cigarettes, showed a nicotine delivery that was comparable to or even higher than that of combustible cigarettes [114-116]. One study on cigalikes and advanced devices has reported plasma nicotine levels of 19 and 35 ng/mL with nicotine liquid concentrations of 12 and 16 mg/mL [114]. However, the puff number of 30 puffs within ten minutes was high compared to similar studies [114]. Plasma nicotine concentration of 20 ng/mL after consumption of a nicotine-salt pod e-cigarette was equal to the nicotine delivery of combustible cigarettes [116]. Nicotine content in the liquid was 59 mg/mL [116]. While this study was performed with experienced dual users of conventional cigarettes and e-cigarettes, inexperienced smokers did not extract the same amount of nicotine from the same e-cigarette brand in another study [117]. These differences in plasma nicotine levels underline the impact of user experience on nicotine delivery.

Assessment of nicotine delivery

Nicotine delivery from e-cigarettes is usually studied with two different clinical study designs that have different aims. In one study design, the participants are asked to use their e-cigarettes or conventional cigarettes following a pre-directed puffing regimen (puff duration, interval, and total number of puffs are specified) [111, 113, 114]. This is done to eliminate factors such as differences in clustering or duration of puffs with the aim to compare different device types, settings, or liquid compositions with each other. The puff volume is still variable. However, in studies predicting aerosol generation by e-cigarettes, the puff volume did not affect amount of generated aerosol [118, 119]. In contrast, puff duration that is correlated with duration of liquid heating is an important factor for liquid aerosolization [118, 119].

During actual consumption, e-cigarette users adjust their puffing behavior, especially puff length [104, 109, 110], to titrate their own nicotine plasma levels. This can be addressed with another study design where the consumers use the e-cigarettes *ad libitum*. Participants can use the studied product freely during a given time frame, e.g., for 90 minutes, to extract the amount of nicotine from the product to their liking [108, 109]. Puff topography can be recorded to gather information on product use [108, 109, 111]. Alternatively, participants could be asked to consume their study product *ad libitum* but only for a predefined short time resembling consumption of a cigarette, e.g., for five minutes [116].

In summary, the study design (e.g., pre-directed vs. *ad libitum* use, experienced vs. unexperienced consumers) should be carefully selected depending on the research question to be addressed.

1.2.2. Emission composition

Analogous to conventional cigarettes, chemical composition of e-cigarette aerosols can be studied with vaping machines. Those are typically close to linear smoking machines (Figure 1, p.18), generating the puff with a piston pump and giving the option to collect particulate and vapor phase with similar methods. Commercial vaping machines are usually equipped with an automated button activator instead of an igniter. Dedicated puffing protocols exist to reflect mouth-to-lung inhalation (i.e., the aerosol is drawn into the mouth, held shortly, and is then inhaled diluted with ambient air) [99]. Protocols from ISO and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) describe a puff volume of 55 mL, puff duration of 3 s, puff interval of 30 s, and a rectangular puffing profile [120, 121]. According to ISO 20768, laboratory air conditions should be kept within 15°C and 25°C room temperature and 40% and 70% rH [120]. In their recommended method No. 81, CORESTA did not define the required temperature and humidity ranges, but stated the allowed variabilities of these parameters [121].

Emission of toxic compounds is usually lower in comparison to conventional cigarettes. Due to the absence of tobacco and the lower temperatures, some major tobacco toxicants like VOCs and PAHs play a minor role in the emissions of e-cigarettes compared with conventional cigarettes. When detected in e-cigarette aerosols, VOCs and PAHs were present in trace amounts [122-125]. However, carbonyl compounds such as formaldehyde, acetaldehyde, acrolein, and propionaldehyde can be formed from PG and VG as displayed in **Figure 4**, as pyrolysis products or as oxidation products catalyzed by free radicals or by the heated metal coil [126-129]. In addition to the liquid bases, flavorings can contribute to formation of carbonyl compounds [130, 131]. Acetaldehyde and formaldehyde are classified as carcinogenic to humans according to EU Regulation No. 1272/2008 on classification, labelling and packaging of substances and mixtures (CLP regulation) [132, 133]. Crotonaldehyde that is also commonly detected in e-cigarette emissions and acrolein have been classified as 2A "possibly carcinogenic to humans" by the International Agency for Research on Cancer (IARC) [134].

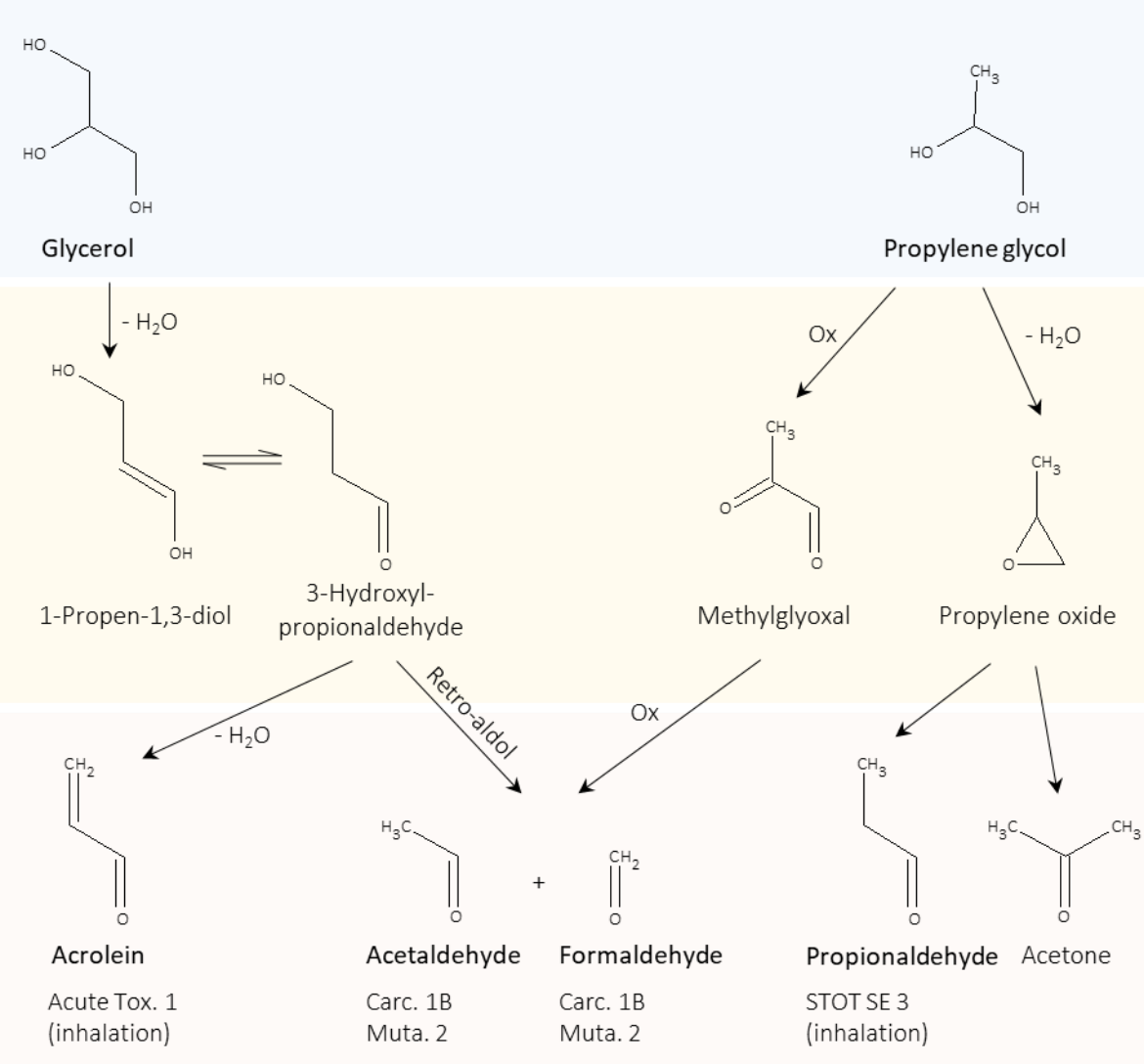

Figure 4. Non-comprehensive summary of pathways for carbonyl compound formation from glycerol and propylene glycol, including relevant hazard classifications (carcinogenic, Carc.; mutagenic, Muta.; acute toxicity, Acute Tox; specific target organ toxicity, single exposure; STOT SE) according to CLP Regulation [132] (adapted from Paine *et al.* [128], Gillman *et al.* [135], Bekki *et al.* [126], and Laino *et al.* [127]).

Carbonyl compounds in e-cigarette emissions are reported in the literature at concentrations that range widely from single-digit values in ng per puff to mid-range values in µg per puff [97, 123, 129, 130, 135-141]. This wide range over four to five orders of magnitude is caused by differences in the used devices and device settings, but also from the analytical methods applied by research groups [137, 142]. A critical factor in carbonyl generation is the heat that is applied as liquid components such as PG and VG are thermally decomposed into carbonyl compounds [129, 137], see **Figure 4**. At certain temperatures, the emissions are perceived as too hot and unpleasant and are accompanied by a high formation of carbonyl compounds [137, 138]. One explanation is the occurrence of dry puffing, meaning that the wick has run dry of liquid temporarily [135, 138]. Temperatures of dry coils are significantly higher than of wetted coils where the energy is consumed by the evaporation process of the liquid components [137, 143]. Formation of carbonyl compounds has been demonstrated by Talih and co-workers to correlate with the applied electrical power when normalized by the surface area of the coil (W/mm^2) also termed “heat flux” [97]. An alternative mechanism behind coil overheating has been proposed by the same authors [144]. They stated that when the heat flux exceeds a critical value, film boiling occurs [144]. This means that a thin film of vapor insulates the coil preventing the energy to be consumed for vapor generation [144]. Instead, the coil overheats causing an increase in carbonyl formation [144]. Further, carbonyl formation was correlated with the amount of generated aerosol and with the puff duration [97, 129, 141].

Formation of carbonyl compounds was accompanied by generation of CO, another product of thermal decomposition of liquid components [141]. Metals like chromium, nickel, copper, and lead have been detected in liquids and e-cigarette aerosols [123, 145-148]. Olmedo *at al.* have analyzed liquids from refill bottles and the same liquid from an e-cigarette tank after use in a machine vaping experiment to sample aerosol. Metals have been found in the generated aerosol and were significantly increased in the used liquid compared with the refill bottle [147]. The heating coils, usually made from metal alloys like kanthal (iron, chromium, and aluminum) and nichrome (nickel and chromium), are one source for metals [147]. Other potential sources are thick wires, brass clamps, solder joints, wick, and sheath [148]. TSNAs have been found in liquids that contained extracts derived from cured tobacco [149-151]. Formation of free radicals was described to be dependent on temperature and liquid composition, including the PG/VG ratio and presence of certain flavoring compounds [152, 153]. Flavoring compounds have the potential to impose a risk, depending on the applied amount and the hazard of the flavoring compound itself or its reaction products [112, 136, 154-156]. Some commonly used flavoring compounds are aldehydes and were shown to form toxicologically relevant acetals with PG and VG [136, 157-159]. In addition, formaldehyde that is generated during aerosol generation forms similar reaction products [160].

1.3. Heated tobacco products

In heated tobacco products (HTP), tobacco is heated instead of directly ignited to produce an inhalable aerosol. The heat can be electrically generated and applied from inside or outside of the tobacco [161, 162]. The heat can also stem from another source like ignited charcoal [163]. In hybrid products between e-cigarette and HTP, liquids with or without nicotine are vaporized and drawn through the tobacco [164, 165]. Tobacco aroma compounds and/or nicotine are extracted *in situ* [164, 165]. The applied temperatures range from approximately 25°C to up to 350°C. In consequence, HTP are heterogenous as a product category and differ in consumer sensations and emission profiles.

1.3.1. Heated Tobacco Products: A Review of Current Knowledge and Initial Assessments

Nadja Mallock, Elke Pieper, Christoph Hutzler, Frank Henkler-Stephani, and Andreas Luch

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MINI REVIEW
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Heated Tobacco Products: A Review of Current Knowledge and Initial Assessments

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The health risks of tobacco smoking have been documented in numerous studies and smoking rates have declined in developed countries over the last 50 years. Today, we know that cigarette smoking is the major cause of preventable deaths due to tobacco smoke induced diseases. As a consequence of an increased awareness of smoking-related health risks, heated tobacco products (HTPs) are marketed as reduced toxicant alternatives to conventional tobacco products. Manufacturers claim that levels of toxicants and hazardous compounds are significantly reduced, implying that inhalation of the modified aerosol is less harmful compared to conventional cigarettes. In this manuscript, previous assessments of HTPs are briefly summarized, including a short discussion on challenges with the adaption of standard analytical methods used for tobacco smoke. The reliability of analytical data is important for risk assessment approaches that are based on reduced toxicant exposure. In order to assess a putative reduction of health risks, an integrated study design is required that should include clinical studies and epidemiology data. One manufacturer applied for a classification as a Modified Risk Tobacco Product (MRTP) in the United States, based on extensive toxicological studies that have also been published. However, data are not yet sufficient for a reliable assessment or recognition of putatively reduced health risks. Challenges regarding a classification in Europe are also discussed briefly in this review.

Keywords: heated tobacco products, emissions, harm reduction, risk assessment, tobacco smoke

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INTRODUCTION

Although most smokers are aware that tobacco smoking is harmful to their health, it is still the leading cause of premature death worldwide and claims the lives of more than 6 million people every year due to cancer, heart disease, stroke, chronic bronchitis, and emphysema (1–4). A recent study has shown that tobacco smoking increases not only the risk for lung cancer, but also for at least 17 different malignant diseases in humans (5); therefore, successful tobacco control can save millions of lives. With the Framework Convention on Tobacco Control (FCTC), the World Health Organization (WHO) has initiated a comprehensive tobacco control strategy (6). Articles 9 and 10 of the FCTC include specific policy measures to curb tobacco use by regulating the ingredients and the emissions of tobacco products. The overall aim is to decrease toxicity, addictiveness, and appeal to consumers. Parties of this convention have committed themselves to restrict the supply and demand of tobacco products through a wide range of policies and measures. Although FCTC was successfully applied to conventional tobacco products, uncertainties remain on how to cover novel products. In October 2018, Conference of Parties (COP) 8 explicitly proposed to extend the scope of the according legislations to Heated Tobacco Products (HTPs) (7).

The chemical complexity of cigarette smoke depends on heating conditions inside the lit cigarette. In a conventional cigarette the burning of tobacco leads to combustion at temperatures up to 700–950°C during puffs (see **Figure 1A**). While combustion is limited to the tip of a burning cigarette, pyrolysis and thermal decomposition occur in the oxygen deficient distillation zone. In this part of the cigarette temperatures decrease from 600 to about 200°C. The majority of smoke toxicants are generated here. Below 350°C, condensation of less volatile compounds generates a dense aerosol consisting of growing droplets and solid particles (8). As a consequence, cigarette smoke consists of "particulate" and "vapor" phases. The mainstream smoke comprises all constituents inhaled during a puff. One way to reduce the exposure to harmful and potential harmful compounds (HPHCs) in the mainstream smoke of tobacco products is to lower the temperature applied to the tobacco. This approach had previously been tried but could not find acceptance on the market as the technology was not yet advanced (9, 10).

Recently, a new generation of HTPs has been introduced to the market which differs widely in product design and temperatures applied to the tobacco. In some devices the tobacco is heated up to 350°C via an electrical heating source (11, 12) or different sources like carbon (13), whereas in other devices vapor is passed through the tobacco and extracts compounds including flavors and nicotine at lower temperatures (14, 15). Three different device designs which are currently present on the market are displayed in **Figure 1B**. These products contain real tobacco that does not undergo a self-sustaining exothermic combustion.

EMISSIONS

In accordance with the principle of temperature dependence of HPHC generation in tobacco products, the question of reduced HPHC levels in the emissions was raised. While manufacturers provided the initial studies (15–18), more and more independent investigations have now been published for commercially available products (19–29). These studies were focused on levels of well-known HPHCs in comparison with other tobacco products. Analyzed HPHCs were adopted from the FDA preliminary HPHC list (30) and recommendations by the WHO Study Group on tobacco product regulation (TobReg) (31). Important carcinogens, such as aldehydes and volatile organic compounds, were found to be reduced by about 80 to over 99% (25). The lowest reduction with only about 80–90% was reported for acetaldehyde, classified as possibly carcinogenic to humans by the International Agency for Research on Cancer (IARC) (32). Toxicants like tobacco-specific N-nitrosamines (TSNAs), formed primarily during curing and processing of tobacco rather than by combustion, were also present in the filler of HTP consumables. However, compared to cigarette mainstream smoke TSNA levels were reduced by about 80–90% (20). Metals like cadmium and mercury are taken up by the tobacco plants and are therefore naturally present in products that contain tobacco (33, 34). Again, levels were reduced in HTP devices. Whereas cadmium was below detection limit, indicating a reduction of over 99% (16, 17), reduction of mercury was ∼75% as published for one device (17). Polycyclic aromatic hydrocarbons (PAHs) and carbon monoxide are typical products of incomplete combustion. Although reduced by more than 90%, they are still present in HTP emissions (17). Other substances, such as propylene glycol, glycidol, acetol, and 2-propen-1-ol have been shown to be elevated in comparison to the combustible reference cigarette in at least one device, due to the higher amount of humectants in the tobacco filler of the HTP consumable (35). Influence on indoor air quality was assessed by the manufacturers and found to be significantly reduced compared to combustible cigarette smoke (14, 36, 37). Concerns for the use in small and poorly ventilated rooms have been raised by an independent group (38).

Reliability and reproducibility of emission data is a crucial factor for a subsequent risk assessment. To benefit most from the increasing pool of independent studies, a common standard for measurements should be agreed on. The first open question arises regarding the machine puffing protocol. There are different arguments for and against various standard protocols, such as ISO (39) or Health Canada Intense (40). Since some of these devices turn off by themselves after a certain time, a smoking regimen with a higher frequency like HCI can help to collect enough material per consumable to pass thresholds set by the analytical instruments. However, the HCI regime could lead to overestimated reductions, due to blocked filter ventilation in conventional or reference cigarettes. Since this modification results in higher toxicant levels in cigarette smoke, the calculated relative reductions of toxicants in the emissions appear bigger. A new puffing protocol, especially tailored for HTPs, would be possible as well. Importantly, these standard protocols do not mimic average smoking behavior and are not meant to provide a realistic estimate of exposure (41). The purpose of defined smoking regimes is to provide standards to compare key parameters of different products when analyzed in different laboratories. However, recent investigations of the puffing topography (42, 43) might suggest further refinements for a better adoption of machine smoking to HTP. ISO/TC126 and CORESTA have started to work on standardized methods.

Since aerosols of HTPs contain a comparatively high proportion of water, standard analytical procedures cannot be easily applied here. Water is trapped on the glass fiber filter and therefore accounts for the total particulate matter (TPM). When the filter is processed further, water loss can occur leading to a reduced analyzed water content. Although not a toxicant, water becomes important when the nicotine-free dried particulate matter, commonly referred to as "tar," is calculated by the subtraction of water and nicotine from TPM (44), though the tobacco industry has developed methods in order to avoid water loss (45, 46). When special equipment is required, implementation as a standard method by independent laboratories becomes difficult. Despite these technical challenges, industry and independent laboratories have come to mostly comparable results when using standard procedures that were designed for the analysis of conventional cigarettes. This indicates that these procedures could be a basis for dedicated analytical standards for HTPs.

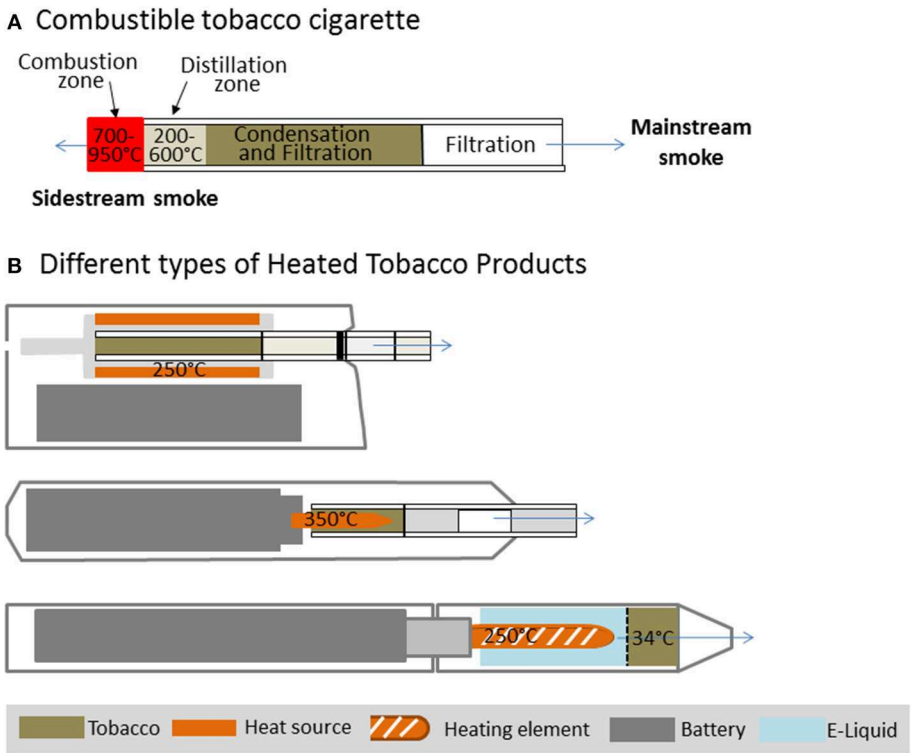

FIGURE 1 | Temperature zones in a combustible cigarette **(A)** in comparison to different Heated Tobacco Products **(B)**.

RISK ASSESSMENT APPROACHES

As discussed, most harmful substances that are known to occur in cigarette mainstream smoke were shown to be lowered by one or two orders of magnitude in HTP emissions. Promoted by the manufacturers, there are discussions if this means a reduction of health risks for HTP consumers followed by controversies whether HTPs can be seen as part of harm reduction strategies. The underlying idea of harm reduction strategies in tobacco control is that the damage caused by tobacco consumption should be at least reduced when it cannot be prevented. Toxicant reduction is not necessarily linked to decreased health risk. Although levels of tar had decreased in combustible cigarettes since the 1950 by nearly two thirds, this was not correlated with corresponding decrease in lung cancer incidences (47). One strategy to assess modified health risks is to compare the tumor potencies of aerosols, as previously applied by Fowles and Dybing to rank the relevant carcinogens and toxicants in cigarette smoke. These calculations are based on individual detection levels in mainstream smoke and on cancer potency factors as indicators of the carcinogenic risk for each smoke constituent (48). The German Federal Institute for Risk Assessment confirmed in its previous study substantially reduced toxicant levels for selected HTPs and provided an initial assessment in 2017 (49). The profound reduction (>99%) of key carcinogens according to Fowles and Dybing, such as benzene and 1,3-butandien, as well as substantial overall reduction of toxicants is expected to affect health risks, if people abstain completely from other tobacco products. Nicotine levels are still in the range of conventional cigarettes, limiting the risk to switch back to conventional smoking tobacco (25). In a detailed modeling assessment, Stephens compared relative harmfulness of different nicotine products with a model based on exposure data and cancer potencies. The calculated lifetime cancer risk of the HTP, using one data set by the manufacturer, was one to two orders of magnitude lower compared to combustible cigarettes but higher compared to e-cigarettes (50). Lachenmeier et al. calculated the combined margin of exposure (MOE) for the HTP and for combustible cigarettes (51). The obtained ratio between exposure and toxicity effect levels, which could be interpreted as a "safety buffer" (52), was 10-fold higher for the HTP as compared to combustible cigarettes (51). As noted by Stephens, these models only consider toxicants levels and neglect particle effects (50). In addition, there is growing consensus that a complete switch to HTP can reduce toxicant exposure, as confirmed in recent investigations on biomarkers of exposure in smokers (53–57). Haziza et al. reported reductions of 51 to 96% for selected HPHC-related biomarkers over a 90-days ambulatory study. However, compliance of participants was decreasing over the ambulatory period, suggesting that relapse to tobacco and/or dual use could counteract potential benefits in real life settings (54). During two 90-days studies, biomarkers of potential harm were additionally assessed (58, 59). The results of longer switching studies to

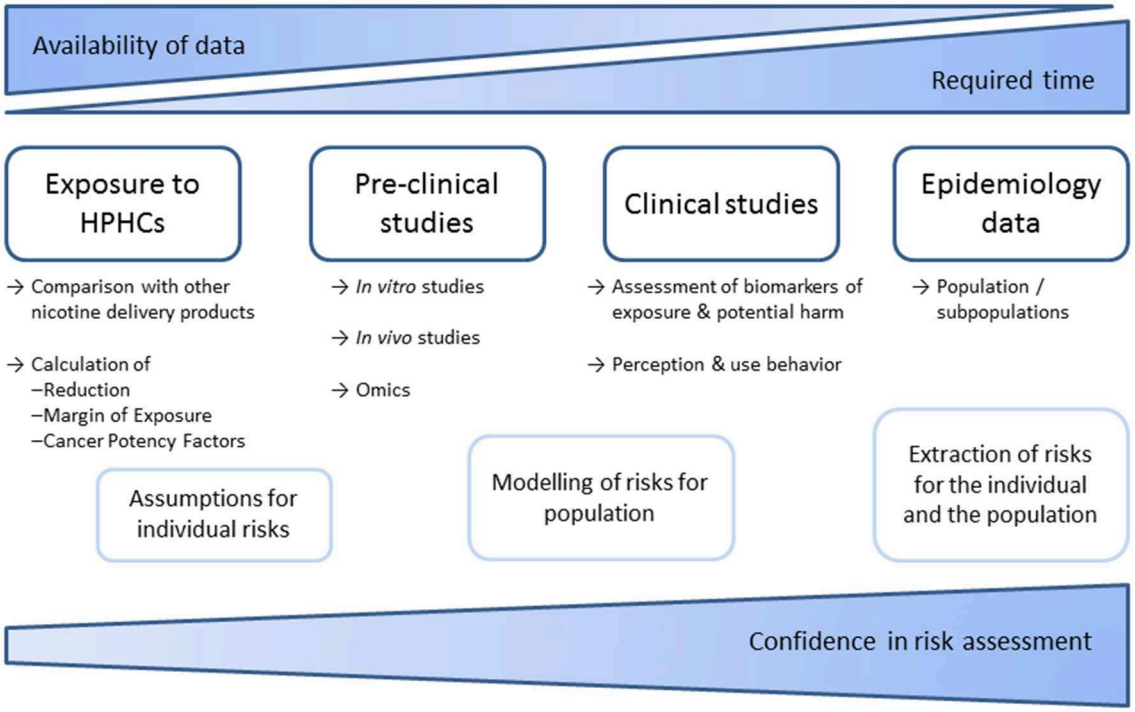

FIGURE 2 | Schematic overview of required data and studies to facilitate risk assessments of tobacco products. Epidemiological data are most conclusive but can usually only be used retrospectively. Therefore, risk assessments rely on models that consider emissions, pre-clinical and clinical studies. Meanwhile numerous studies on smoke chemistry and *in vitro* toxicology have been published by industry and independent researchers. In contrast, *in vivo* and clinical studies are far more complex. No sufficient independent data are available.

detect significant reductions of biomarkers of potential harm are anticipated (60).

In the United states, the Family Smoking Prevention and Tobacco Control Act (61) requires tobacco products to not only "*significantly reduce harm and the risk of tobacco-related disease to individual tobacco users*" but also to "*benefit the health of the population as a whole taking into account both users of tobacco products and persons who do not currently use tobacco products*" in order to market that product with modified risk claims in the United States. The required scientific evidence for defined claims and additional data that have to be provided by the applicant are described by the FDA in detail in a guidance document (62). Scientific standards for analysis of potential Modified Risks Tobacco Products were also outlined by the Institute of Medicine in 2012 (63). Required data (summarized in **Figure 2**) include a comprehensive analysis of smoke chemistry (64) as well as data on specified biomarkers of exposure. There is a framework for preclinical studies, proposing *in vitro* tests of genotoxicity, oxidative stress, and inflammation. The *in vitro* test battery comprising assays for bacterial mutagenicity, mammalian cytogenetics/mutation, and mammalian cytotoxicity, that has been suggested by a CORESTA task force in 2004 (65), has been conducted by the manufacturers (17, 18, 66–70). Some *in vitro* tests can specifically address smoking related adverse effects, as biphasic culture of airway epithelial cells or assays on endothelial activation as conducted by the manufacturers (13, 71–74) and independent researchers (75, 76). Further, 3D *in vitro* cultured lungs tissues are now available by several commercial suppliers. Consequently, the necessity for animal testing of tobacco products should be questioned, in line with a general shift of focus in modern toxicology (77). In some countries including Germany, animal studies have been prohibited for tobacco products. However, animal studies have been conducted by the tobacco industry (78–81) and independent researchers (82). To address public health questions, population models have been applied (83–86) and publically discussed (87).

In Europe, toxicological assessments of tobacco products are aimed to exclude elevated risks in relation to conventional products, but not to confirm less hazardous product properties. As long as relevant adverse effects cannot be excluded, even modified health risks still remain an issue of concern. In contrast to the United States, products can be placed on the market more easily. Consumers who use these products need to accept all characterized and not yet identified health risks. Also manufacturers might attempt to gain classification as "smokeless tobacco," resulting in less stringent health warnings. In public perception, this could probably be understood as an official acknowledgment of reduced health risks. Such acknowledgment would be premature from the perspective of risk assessment. In the USA, the assessment framework is required to acknowledge reduced/modified risks, if manufacturers can support their claims. Consequently, additional issues, as for example risk

perception and communication, behavioral assessments of addictiveness or clinical studies (63) need to be considered.

In May 2017, one manufacturer submitted a Modified Risk Tobacco Product Application (MRTPA) for his HTP (88) and in January 2018, the Tobacco Product Scientific Advisory Committee (TPSAC) met to give a recommendation. Due to the lack of human studies, TPSAC was not convinced to support the statement "*Scientific studies have shown that switching completely from cigarettes to the IQOS system can reduce the risks of tobacco-reduced diseases*," although potential is seen. The relevance of the animal studies to human smokers has been questioned (89). Two 90-days studies as mentioned above (58, 59) did not demonstrate a relevant reduction in biomarkers of potential harm in regard to inflammation and lung function (90). This could also be linked to the continual inhalation of nicotine and remaining toxicants. Reductions of biomarkers of potential harm were also low in the smoking abstinence groups, possibly due to the short study period. Biological relevance needs to be demonstrated with longer exposure studies. However, biomarkers of exposure that have been assessed in various studies were shown to be reduced similarly to cessation level (35), especially markers that are relevant for carcinogenic risks. The less strong claim "*Switching completely to IQOS presents less risks of harm than continuing to smoke cigarettes*" has therefore been supported by about half of the committee members (89). While the evidence has mostly been seen as strong enough to support a reduced exposure claim, the link to morbidity and mortality has not been seen to be adequately demonstrated (89). The final decision on the MRTPA has not been made by the FDA yet, however the first HTP was authorized in April 2019 for sale, without modified risk status. In Europe, it is widely accepted that current HTPs do not bear additional or other health risks in relation to conventional products. European legislation does not define a modified risk classification. On the contrary, information on the product and package, as well as presentation must not imply reduced hazards compared to any other tobacco product. Although a risk-benefit assessment is required for new tobacco products, permission on the market does not depend on modified risks.

Although a 99% reduction of some major carcinogens is expected to affect health risks, the magnitude or relevance of such putative reduction is not yet clear. A benefit is likely seen for especially the subset of long-term smokers that are unable to quit or to switch to another nicotine source with less HPHC exposure. However, referring back to the tumor potency models, it should be kept in mind that substantial and relevant health risks are still present. Consequently, HTPs should not be the first option to decrease smoking-associated harm.

AUTHOR CONTRIBUTIONS

NM prepared the draft manuscript. EP and FH-S contributed sections to the draft. NM, EP, CH, FH-S, and AL created the concept for this article and contributed to manuscript revision and approval of the final version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.4. Objective

Emerging nicotine delivery products can have an influence on health risks of individuals and the whole population, as discussed under 1.3.1. Heated Tobacco Products: A Review of Current Knowledge and Initial Assessments [166]. Generation and release of toxic substances by ANDS can be drastically reduced compared with tobacco cigarettes (as discussed under 1.2.2. and 1.3.1.). A complete switch from tobacco cigarettes to such products reduces the exposure and thus, likely also the health risks associated with tobacco smoking [167-169]. Consequently, they have the potential to reduce smoking-associated harm.

For assessment of effects of ANDS on the population level, further aspects have to be considered. ANDS that deliver a high amount of nicotine could induce addiction in non-smokers, potentially leading to cigarette smoking (gateway effect), or promote a relapse in former smokers [170]. In conclusion, critical factors for a potential harm reduction are the exposure to harmful substances that should be substantially reduced (in comparison to cigarette smoking), and the nicotine delivery that should be high enough to facilitate a complete switch but should not induce addiction in naïve users. Other factors such as attractiveness that are relevant for product uptake have not been addressed with this work.

Nicotine delivery and emission of hazardous substances can differ throughout product categories, meaning that not all e-cigarettes are the same and can have completely different evaluations regarding personal health risks and public health effects. This also applies to HTPs and other ANDS. It is not feasible to conduct clinical studies for every product to perform a risk assessment. Thus, as already outlined above in Chapter 1.3.1., a first assessment should be based on data that can be easily acquired for many products such as emission chemistry data on nicotine and hazardous substances. Ideally, this initial assessment could then be used to decide on the necessity for further investigations that are cost-, work-, and time-intensive. Accordingly, emission chemistry data should be reliable and predictive for subsequent effects.

Therefore, the main subject of this thesis is the initial assessment approach of emerging ANDS on basis of their emission chemistry with two central aims:

- Establishment of methods for characterization of delivery of nicotine and hazardous substances from ANDS:
    - While standardized analytical methods are available for conventional cigarettes, chemical assessment of novel ANDS categories usually starts without standardized protocols or reference materials. Thus, analytical methods to assess relevant analytes in mainstream emissions of HTPs are established in this thesis based on existing standards for combustible cigarettes.

- As nicotine delivery of a product is an important predictor of addictiveness, the relationship between nicotine delivery of a novel e-cigarette in a clinical study and the nicotine concentration in machine generated emissions is investigated. This is an important step in establishing emission nicotine levels as a predictor for nicotine delivery at stage of consumption.
- Chemicals in cigarette smoke have been intensively studied and are well characterized. However, emerging products and new materials might inherit risks due to exposure to unknown substances. A method is established that can be used to characterize potentially unknown hazardous thermodegradation products from new materials used in ANDS.

- Characterization and discussion of emissions of a novel pod e-cigarette and HTPs in context of an initial risk assessment:
  - Levels of two groups of major contributors to cancer risk in tobacco smoke, VOCs and carbonyl compounds, are determined in HTP mainstream emissions. These data are used to discuss the potential exposure reduction and putative health effects of the products.
  - Nicotine delivery of a pod-type e-cigarette containing nicotine salts is studied. Potential addictiveness and satisfaction for addicted consumers are estimated based on these results.

2. Results

Experimental work and outcomes are presented in six separate parts that are grouped according to the studied products, e-cigarettes and HTP:

- 2.1. Electronic cigarettes
  - 2.1.1. Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols

    Study aim: Machine generated emissions of nicotine and carbonyl compounds are characterized for a pod e-cigarette that contains nicotine salts. Influence of a product modification by the manufacturer on the product emissions is investigated. Further, methodological problems when studying e-cigarettes with a rectangular rather than a round mouth-piece shape are addressed. This chapter has been published in *Archives of Toxicology* [171].

  - 2.1.2. Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans-3'-hydroxycotinine* by LC-MS/MS: Method development and validation for human plasma

    Study aim: A method is developed and validated for the determination of nicotine and its main metabolites cotinine and *trans*-3'-hydroxycotinine from human plasma. The method should enable high sensitivity despite the rapid sample preparation step allowing a high throughput. Reliability is assured with a validation compliant with biomedical guidelines. This chapter has been published in *Journal of Chromatography B* [172].

  - 2.1.3. Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after pod modification

    Study aim: Nicotine delivery of the e-cigarette studied under 2.1.1. and the influence of the prior investigated product modification are determined at stage of consumption in a clinical study. Focus is set on the acute phase that is assumed to be of special relevance for addiction. This chapter has been published in *Scientific Reports* [173].

- 2.2. Heated tobacco products
  - 2.2.1. Levels of selected analytes in the emissions of “heat not burn” tobacco products that are relevant to assess human health risks

    Study aim: Selected emissions of a novel HTP are determined to support an initial science-based risk assessment. For this, analytical methods are adapted and established for this product group. This chapter has been published in *Archives of Toxicology* [174].

  - 2.2.2. Levels of selected analytes in the emissions of a heated tobacco product with external heating of the tobacco

    Study aim: Established methods are tested with a second HTP that has a different tobacco heating concept. Data on emissions of toxicological relevance for the second product are collected. This chapter contains previously unpublished results.

  - 2.2.3. Online-coupled pyrolysis gas chromatography as a useful tool to identify unknown thermal degradation products from materials in heated tobacco products

    Study aim: Identification of potential new risks, thermodegradation products of a new filter material that is used in consumables of a HTP are characterized. For this, a method based on online-coupled pyrolysis gas chromatography is applied. This chapter contains previously unpublished results.

The central aims of this thesis are addressed and discussed in an integrated discussion in Chapter 3.

2.1. Electronic cigarettes

2.1.1. Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols

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Online Supplementary Material is presented in **Annex I**.

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ANALYTICAL TOXICOLOGY

Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols

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Abstract

The popularity and the high nicotine content of the American pod e-cigarette JUUL have raised many concerns. To comply with European law, the nicotine concentration in the liquids of the European version, which has been recently released on the market, is limited to below 20 mg/mL. This limit can possibly be circumvented by technological adjustments that increase vaporization and consequently, elevate nicotine delivery. In this study, we compare vapor generation and nicotine delivery of the initial European version, a modified European version, and the original American high-nicotine variant using a machine vaping set-up. Additionally, benzoic acid and carbonyl compounds are quantified in the aerosol. Further, concentrations of nicotine, benzoic acid, propylene glycol, and glycerol, along with the density and pH value of JUUL e-liquids have been assessed. Whereas the initial European version did not compensate for the low nicotine content in the liquid, we provide evidence for an increased vaporization by the modified European version. As a consequence, nicotine delivery per puff approximates the American original. Notably, this is not associated with an increased generation of carbonyl compounds. Our data suggest a similar addictiveness of the enhanced European version and the original American product.

Keywords JUUL · Electronic cigarette · Nicotine salt · Nicotine delivery · Vapor chemistry · Benzoic acid

Introduction

Electronic cigarettes have been and are still at the center of controversies among researchers and policy makers. There is growing consensus that the exposure to carcinogens and hence toxicological risks are markedly reduced when compared to combustible tobacco cigarettes (Stephens 2018), where combustion and pyrolysis of organic material lead to the formation of toxicologically relevant substances (Rodgman and Green 2003). Yet, carbonyl compounds, such as acetaldehyde or formaldehyde, have also been found in e-cigarette vapor under dry-puff conditions. When used under normal conditions, much lower levels are detected compared to cigarette smoke though (Farsalinos and Gillman 2018; Goniewicz et al. 2014; Hutzler et al. 2014). Carbonyls can be formed from the liquid components glycerol (vegetable glycerol, VG) or propylene glycol (PG) by thermal decomposition (Gillman et al. 2016; Paine et al. 2007). On the other hand, novel risks may arise from constituents that are untypical for conventional cigarettes (Erythropel et al. 2019b; Kaur et al. 2018). Despite the confirmed reduction of the long known and established toxicants, possible health disturbances of e-cigarette consumption, such as the impact of nicotine on brain development (Dwyer et al. 2008; Smith et al. 2015) and the cardiovascular system (Buchanan et al. 2019), need to be further investigated and assessed. Conversely, also assumed benefits on the population level need to be clarified. For example, it is criticized that smokers who switch to e-cigarettes do not quit nicotine consumption but use e-cigarettes for a longer term. A gateway effect is debated, but more data are required to clarify this

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assumption (Conner et al. 2018; Etter 2018; Kandel and Kandel 2015; Liu et al. 2020; Watkins et al. 2018).

The product spectrum of e-cigarettes is rapidly expanding. Currently, there are two major developments in the field: First, "open systems" that allow the consumer to adjust vaporization power settings and the nicotine concentration in the liquid individually, and second, "pod systems" that already contain the coil, the wick and a small liquid reservoir. These latter devices are comparatively simple and easy to use. Pod systems usually contain highly concentrated nicotine salt formulations and have recently been demonstrated to deliver nicotine nearly equally efficient as combustible cigarettes (Bowen and Xing 2014; O'Connell et al. 2019). The high nicotine content of up to 5% (approximately 58 mg/mL), the resulting high pH value of the vapor and the high proportion of free-base nicotine would normally lead to airway irritation (Duell et al. 2018). Weak organic acids, such as benzoic or salicylic acid, are being supplemented to adjust the pH value and the amount of free-base nicotine to a level that is more tolerable for the consumer (Bowen and Xing 2014; Duell et al. 2018).

E-liquids of the brand JUUL contain benzoic acid and high nicotine concentrations of up to 5%. The brand had reached a market share of about 40% in the US by the end of 2017 (Huang et al. 2019) with high popularity among adolescents (Hammond et al. 2019; Krishnan-Sarin et al. 2019). This is likely due to viral marketing and the spreading of the product via social media and YouTube (Allem et al. 2018; Brett et al. 2019; Chu et al. 2018; Czaplicki et al. 2019; Kavuluru et al. 2019; Ramamurthi et al. 2019). The product design is flat and the vapor generation is low, which allows unsuspicious "stealth" vaping that even went viral as an internet challenge (Ramamurthi et al. 2019). Evidence for nicotine dependence in adolescent pod users has been shown in a pilot study (Boykan et al. 2019). The recent e-cigarette innovations have led to a "public health epidemic" in the US, as stated by the Surgeon General (U.S. Surgeon General 2018).

In 2018, JUUL was also introduced in Germany and was required to comply with the European Tobacco Product Directive 2014/40/EU (TPD), in respect to the upper limit of 20 mg/mL for nicotine (European Parliament 2014). Consequently, a lower nicotine delivery could be expected for the European version, but no data are yet available. Some research groups have already characterized the US-American variant (Duell et al. 2018; Erythropel et al. 2019a; Pankow et al. 2017; Reilly et al. 2019; Talih et al. 2019). The nicotine content in the aerosol was comparable to combustible cigarettes, but formation of carbonyl compounds was low as expected for low power vaporizers (Reilly et al. 2019; Talih et al. 2019). We have hypothesized that the manufacturer might increase the vapor generation of the European version to compensate for the low nicotine content in the liquid. In fact, an improved version of European JUUL, referred to as "Turbo" by JUUL employees (Mahase 2019), has been recently introduced in Germany and prompted concerns of an increased addictiveness. In this study, we aim to set a reference point for nicotine and toxicant levels in the aerosol of the initial and the modified version of JUUL. Technical aspects for vaping machine experiments are also briefly discussed. Therefore this study provides a scientific ground for the monitoring of current and further directions of product development.

Methods

E-cigarettes and pods

The European devices and differently flavored pods were purchased in local stores in Berlin and Sigmaringen, Germany, and online. The US-American variant was purchased in Tempe, Arizona.

Chemicals and standard substances

Used solvents or chemicals were of analytical or higher purity grade. 2-Propanol containing 0.3 g/L *n*-heptadecane, 2 g/L ethanol as internal standards and (*S*)-nicotine salicylate were purchased from LGC Standards (Teddington, UK), acetonitrile, sodium chloride, and orthophosphoric acid (85%) from Merck KGaA (Darmstadt, Germany). 2,4-Dinitrophenylhydrazine (moistened with 33% water) was bought from PanReac AppliChem (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane, sulfuric acid (99.999%), (*S*)-nicotine, benzoic acid, benzoic acid-d_5, and the 2,4-dinitrophenylhydrazone (DNPH) derivatives of acetaldehyde, acetone, acrolein, and formaldehyde were purchased from Sigma-Aldrich (Taufkirchen, Germany). Dimethyl sulfoxide was purchased from Honeywell Riedel-de-Haën (Seelze, Germany). Ultrapure water was prepared with a Milli-Q Integral Water Purification System (Merck KGaA, Darmstadt, Germany).

Aerosol generation

Aerosols were generated in two different laboratories, hereafter referred to as "lab A" (BfR, Berlin, Germany) and "lab B" (CVUA Sigmaringen, Sigmaringen, Germany). Both laboratories used a standard linear smoking machine that was designed for e-cigarettes (LM4E with PM1 piston pump, Borgwaldt, Hamburg, Germany). Experiments were performed according to CORESTA Reference Method 81 (CORESTA 2015) for the puffing regimen: 55 mL puff volume, 3 s puff duration, 30 s puff frequency, and rectangular puffing profile. E-cigarettes were placed in an angle of − 15°

from a horizontal position. Except for carbonyl analysis, sessions of 20 puffs were taken with a clearing puff without e-cigarette at the end of each session. Between sessions, the liquid was allowed to cool down for approximately 10 min. The batteries were recharged after 8 and 6 sessions for the initial and the modified pods, respectively. Lab A compared two different custom adapters for a tight placement of the angular shaped e-cigarette mouth-piece on the filter holders as displayed in Fig. 5 in the Supplementary Material. One adapter was self-made with a heat shrinkable tubing (cross-linked polyolefin, HStronic GmbH, Schwäbisch Hall, Germany) that was prepared once and reused in combination with tape sealing (Parafilm, Bemis Company, Neenah, WI, USA). The second adapter was purchased from Borgwaldt (Hamburg, Germany) and used without additional tape sealing. Lab B used only the mouth-piece from Borgwaldt.

Determination of liquid consumption and total particulate matter (TPM), water and nicotine in the aerosol

TPM was collected on Ø 44 cm glass fiber filters (Borgwaldt, Hamburg, Germany). The filters in the filter holders and the e-cigarettes with pods were weighed before and after each session on analytical scales (LE225-0CE in lab A and CP225D-0CE in lab B, both Sartorius, Göttingen, Germany). TPM was calculated with the weight gain of the filters according to ISO 4387 (2019), consumption of the e-liquid with the weight loss of the liquid. Filters were extracted with 20 mL isopropanol containing internal standards (0.3 mg/mL *n*-heptadecane, 2 mg/mL ethanol) on a horizontal shaker (lab A: 3005, GFL, Burgwedel, Germany; lab B: SM-30 Control, Edmund Bühler, Hechingen, Germany) for 30 min at 80–100 rpm. The extracts were used for the determination of nicotine and water. Nicotine was quantified by gas chromatography with flame ionization detection (GC/FID). Lab A used a 6890 series from Agilent Technologies/Hewlett Packard (Agilent Technologies, Waldbronn, Germany) with a constant flow of 1.3 mL/min hydrogen (purity 99.999%, Linde, Pullach, Germany) on an HP-5 ms capillary column (30 m length, 250 µm inner diameter, 0.25 µm film thickness, 3 m pre-column, Agilent Technologies, Waldbronn, Germany). The temperature program started with 5 min at 100 °C, followed by a 30 °C/min ramp to 325 °C with 3.50 min hold. 1 µL filter extract was injected into a split/splitless injector at 250 °C and split ratio of 1:5 was used. FID was operated at 290 °C with a hydrogen flow of 30 mL/min, air flow of 300 mL/min, and a nitrogen (purity 99.999%, Linde, Pullach, Germany) make up flow of 20 mL/min. Lab B analyzed nicotine with flame ionization detection at 300 °C (7890A, Agilent Technologies, Waldbronn, Germany; 30 mL/min H_2 flow, 99.999%; 400 mL/min air flow; 15 mL/min make up flow, N_2, 99.999%; Air Liquide, Paris, France) and water with thermal conductivity detection at 250 °C (Agilent Technologies, Waldbronn, Germany; 15.5 mL/min reference flow, 5 mL/min combined flow) in one run after separation with a 7890A series gas chromatograph (Agilent Technologies, Waldbronn, Germany). 1 µL extract was injected into a split/splitless injector at 250 °C in splitless mode. Separation for nicotine analysis was performed by using an Rtx-VMS column (30 m × 0.530 mm, 3 µm film thickness, Restek GmbH, Bad Homburg, Germany), and for water an HP Plot Q column (30 m × 0.530 mm, 45 µm film thickness, Agilent Technologies, Waldbronn, Germany).The oven program started at 75 °C for 0.5 min, heated with a rate of 50 °C/min to 165 °C and held for 3 min, heated with 50 °C/min to 225 °C, held for 5 min, before it cooled down at 50 °C/min to 75 °C, followed by a 1 min hold. Flow rate of helium carrier gas (99.999%, Air Liquide, Paris, France) was 4.240 mL/min.

Determination of carbonyl compounds

Carbonyls were analyzed as described previously (Mallock et al. 2018) with liquid chromatography and UV detection on an RP-Amid column (Ascentis, 150 × 2 mm, 3 µm, Supelco, Bellefonte, PA, USA). Fractions of 40 puffs each were drawn through impingers that contained 35 mL of 2,4-dinitrophenylhydrazine (3.4 mg/mL in 45% acetonitrile with 0.35% orthophosphoric acid) each. After two clearing puffs, the content of both impingers was combined and incubated at room temperature for 30 min before reaction was stopped by addition of 2 mL tris(hydroxymethyl)aminomethane (tris) solution (16 mg/mL in 80% acetonitrile) to 8 mL of the sample. Calibration standards for carbonyl-DNPH-derivatives were diluted with the same DNPH/tris solution mixture to mimic effects of excess DNPH on the UV spectra.

Determination of benzoic acid in liquids and aerosol

Benzoic acid was quantified using headspace-solid phase microextraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS). 60 mg of sample liquid or self-prepared standard liquid (20 mg/mL nicotine in PG/VG 50:50 (w/w)) was weighed into 20 mL headspace vials and dissolved in 5 mL saturated sodium chloride solution containing 0.5 M sulfuric acid. 50 µL of isotope-labeled internal standard solution (8 mg/mL benzoic acid-d_5) and/or calibration standard solution (4, 10, 15, 20 and 30 mg/mL benzoic acid) in DMSO were added and mixed. For analysis of benzoic acid in the aerosol, vaped filters were transferred into 20 mL headspace vials. For calibration, blank filters were used in combination with 40 mg or 80 mg standard liquid. Standard solutions were directly pipetted on the filter, followed by mixing with 5 mL saturated salt solution containing 0.5 M sulfuric acid. SPME was automated on

an MPS2-XL autosampler (Gerstel, Mühlheim, Germany) with an incubation temperature of 80 °C and 1 min incubation time prior to 50 min headspace extraction by a polydimethylsiloxane/divinylbenzene fiber (Supelco, Bellafonte, PA, USA) with 250 rpm shaking only for the filter samples. The fiber was injected into a cooled injection system (CIS) 4 (Gerstel, Mühlheim, Germany) and desorbed for 5 min at 250 °C and a 1:50 split ratio. The GC 6890A (Agilent Technologies, Waldbronn, Germany) was equipped with a 30 m HP-FFAP capillary column with 250 µm inner diameter and 0.25 µm film thickness (Agilent Technologies, Waldbronn, Germany). After 5.5 min at 60 °C, the GC oven ramped with 15 °C/min to 240 °C and held for 15 min. The helium (purity 99.999%, Linde, Pullach, Germany) carrier gas flow was constant at 1 mL/min. The mass selective detector MSD 5975C (Agilent Technologies, Waldbronn, Germany) was equipped with an electron impact ion source (Agilent Technologies, Waldbronn, Germany) and operated with an ionization energy of 70 eV using a combined selected ion monitoring (SIM) and scan mode with a mass range from 29 to 300 m/z. Benzoic acid was quantified with the ion masses of 77 m/z and qualified with 105 m/z and 122 m/z. The internal standard benzoic acid-d_5 was quantified with 82 m/z and qualified with 110 m/z and 127 m/z. Dwell time was 15 ms for each ion. Optimization of extraction parameters is summarized in the Supplementary Material.

Determination of density, pH value and nicotine content of the e-liquid

Liquids from the same batch were pooled for direct determination of density with an oscillating U-tube (DMA 500, Anton Paar, Graz, Austria). For quantification of the nicotine content in liquids, 300 mg liquid was diluted in 10 mL isopropanol with internal standards (0.3 mg/mL *n*-heptadecane, 2 mg/mL ethanol) and analyzed with the above mentioned GC/FID method in lab B. The pH value of a 1:20 dilution of liquids in ultrapure water was directly measured with a pH meter (765 Calimatic; Knick, Berlin, Germany).

Determination of propylene glycol and glycerol content of the e-liquid

E-liquids were analyzed by diluting a sample solution of approx. 5 mg/mL (precisely weighed) with methanol. The resulting solution was diluted by 1:1 with the internal standard solution containing 5 mg/mL 1,4-butanediol in methanol. 1 µL aliquot of this sample solution was injected into the split/splitless injector and analyzed by means of GC/FID. GC/FID analysis was performed on an Agilent 7890A gas chromatograph equipped with an FID detector and an autosampler (Agilent Technologies, Waldbronn, Germany). Separation was achieved on an HP-FFAP (25 m × 0.32 mm i.d. × 0.52 µm film) capillary column (Agilent Technologies, Waldbronn, Germany). GC/FID conditions were as follows: split mode, split ratio: 1:40; injector temperature: 230 °C; nitrogen (99.999%; Air Liquide, Paris, France) as carrier gas at a constant pressure of 0.7 bar. FID was operated at 250 °C (30 mL/min H_2 flow, 99.999%; 400 mL/min air flow; 30 mL/min make up flow, N_2, 99.999%; Air Liquide, Paris, France). The oven program started at 70 °C, held for 4 min. The temperature was raised by 10 °C/min up to 220 °C and held for 7 min, followed by a ramp of 30 °C/min to 70 °C. Total run time was 31 min.

Characterization of the pod construction

Resistance was measured between the connectors at the bottom of the pods with a 2010 DMM ohmmeter (PeakTech, Ahrensburg, Germany). For FT-IR analysis, the wick was removed from the pod, washed twice with ethanol, dried at 80 °C (FED 240, Binder, Tuttlingen, Germany), and analyzed with attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) spectroscopy using a Nicolet 6700 spectrometer (Thermo Electron Corporation, Madison, WI).

Results

Description of the product

The JUUL device consists of a flat and elongated battery with contacts to connect to the particular pods as shown in Fig. 1. The prefilled and disposable pods are composed of an e-liquid tank, including coil and wick, and a rectangular mouthpiece. The pods are marketed in four-packs and are declared to contain approximately 0.7 mL liquid with formerly 20 mg/mL (referred to as "initial" in this publication) and now 18 mg/mL and 9 mg/mL nicotine (referred to as "modified"). The modified JUUL version has been launched in Germany in summer 2019, and could still be included in our study. Vegetable glycerol (VG), propylene glycol (PG), nicotine, benzoic acid and "aromas" are listed as ingredients. The packets contain health warnings and hazard pictograms and refer to the product as "alternative for adult smokers".

Chemical characterization of JUUL pods and aerosol

Different analytical assessments have been performed in the liquid and the aerosol of JUUL pods with the aromas "Rich Tobacco" (initial and modified pods in comparison), "Royal Creme", "Mint", "Mango", and "Apple" (initial pods). The results are shown in Table 1. Although declared as 20 mg/mL, nicotine content was found to be below 18 mg/mL in the initial pods. Thus, the modified European pods contained the same amount of nicotine as the initial ones. Density,

Fig. 1 Photography of a JUUL e-cigarette with a battery and differently flavored pods

composition of the liquid basis, pH values, and amount of benzoic acid did not vary significantly between different aromas of the initial pods. Modified European JUUL generated more TPM (as marker for vapor generation) as the initial European version. The molar ratio of nicotine to benzoic acid decreased in both liquid and vapor of the improved European version, implying that more benzoic acid is now being applied. The pattern of aldehyde formation changed with the alteration of the pod design: The generation of acetone increased whereas the generation of acetaldehyde and formaldehyde decreased. For formaldehyde, the high standard deviation is likely due to inter- and intra-device variabilities of carbonyl generation. As discussed in the Supplementary Material, the concentrations of all other analytes were close to its analytical thresholds, what could have been an additional factor for high deviations. Furthermore, the amount of water in the vapor has been assessed for 10 initial Rich Tobacco JUUL pods. The mean and standard deviation of the first 160 puffs were 0.25 ± 0.08 mg water/puff.

Comparison of European JUUL pods with the US-American version

As visualized in Fig. 2, the US-American JUUL device released 1.4 ± 0.4 mg TPM per puff, resulting in a similar vapor generation compared to the initial European JUUL. The nicotine delivery was with 72 ± 25 µg per puff approximately threefold higher. This correlates with a threefold higher nicotine content in the liquid. The vapor generation of the European modified JUUL pods was more than doubled compared to both the European initial pods and the US-American version. Accordingly, the nicotine delivery of modified European JUUL approximated to the high-nicotine US-American variant. The resistance between the connections of the pods, reflecting the resistance of the coil, has been measured and ranged between 1.6 and 1.7 Ω for all three variants. As shown in Fig. 3, the wick material has been replaced in the modified JUUL variant. The change in the material has been confirmed with ATR-FTIR. Spectra are displayed in the Supplementary Material.

Intra-device variability of nicotine delivery

According to European tobacco legislation, E-cigarettes need to deliver nicotine at consistent levels. We have therefore tested whether JUUL complies with this requirement. As illustrated in Figs. 6 and 7 in the Supplementary Material, the aerosol generation of JUUL e-cigarettes varied significantly over all fractions. The continuity of nicotine delivery was assessed in light of the intra-device variability. For each pod analyzed, the mean nicotine delivery for the first 8 fractions was calculated. The difference of each single value to the mean was calculated in percent. The highest difference to the mean was set as maximum deviation for the corresponding pod type in our experiments. This reflects the intra-device variability. Only the first 8 fractions of 20 puffs each were regarded as intended use. Out of 10 initial JUUL pods for each laboratory, the maximum deviation was + 31% and + 79% as determined by lab A and lab B, respectively. Out of the 20 pods, 17 had a maximum deviation above an exemplary threshold of 15% that might be used to define a consistent nicotine delivery. The maximum intra-device deviation out of 6 modified pods was − 50% and − 45% for 18 mg/mL and 9 mg/mL nicotine pods, respectively. For all modified pods analyzed, the maximum deviation was found for the first fraction. All 12 pods had a maximum deviation above 15%. When this fraction was left out from the calculation, the maximum deviation was still characterized as − 45% and − 39% for 18 mg/mL and 9 mg/mL nicotine pods, respectively, with 6 out of 12 pods above 15%.

Table 1 Chemical characterization of JUUL liquids and aerosol

Flavor	Rich tobacco	Rich tobacco	Rich tobacco	Royal Creme	Apple	Mango	Mint
Pod design	Initial	Modified	Modified	Initial	Initial	Initial	Initial
Declared nicotine concentration (mg/mL)	20	18	9	20	20	20	20
Characterization of liquids							
Measured nicotine concentration (mg/mL)	17.20 ± 0.13 (3 pods)	17.69 ± 0.09 (3 pods)	9.03 ± 0.14 (3 pods)	17.41 ± 0.05 (3 pods)	17.40 ± 0.28 (3 pods)	17.78 ± 0.14 (3 pods)	17.26 ± 0.21 (3 pods)
Density (g/cm^3)	1.16	1.16	1.16	1.18	1.18	1.18	1.18
Liquid basis (g/100 g)	PG: 26.0 ± 1.6 VG: 56.8 ± 4.0 (5 pods)	PG: 24.4 ± 2.1 VG: 55.8 ± 4.9 (3 pods)	PG: 27.6 ± 0.1 VG: 61.2 ± 0.8 (3 pods)	PG: 23.8 ± 1.5 VG: 64.7 ± 3.5 (3 pods)	PG: 24.0 ± 1.6 VG: 62.5 ± 4.4 (3 pods)	PG: 24.9 ± 0.2 VG: 65.8 ± 0.7 (3 pod)	PG: 23.6 ± 0.4 VG: 65.6 ± 1.2 (4 pods)
pH value (of 1:20 dilution in ultrapure water)	5.51 (1 pod)	5.42 (1 pod)	5.40 (1 pod)	5.42 (1 pod)	5.74 (1 pod)	5.56 (1 pod)	5.52 (1 pod)
Benzoic acid (mg/mL)	9.64 ± 0.05 (3 pods)	12.67 ± 0.38 (3 pod)	7.02 ± 0.21 (3 pods)	9.24 ± 0.04 (3 pods)	8.82 ± 0.59 (3 pods)	9.24 ± 0.09 (3 pods)	9.17 ± 0.02 (3 pods)
Molar ratio (Nicotine:Benzoic acid)	1:0.7	1:1.0	1:1.0	1:0.7	1:0.7	1:0.7	1:0.7
Characterization of aerosol							
TPM (mg per puff) Mean of the first 160 puffs	1.6 ± 0.4 (20 pods)	3.7 ± 0.7 (6 pods)	3.7 ± 0.7 (6 pods)	1.8 ± 0.5 (2 pods)	1.8 ± 0.3 (2 pods)	1.8 ± 0.3 (2 pods)	1.9 ± 0.3 (2 pods)
Nicotine (µg per puff) Mean of the first 160 puffs	23 ± 5 (20 pods)	61 ± 12 (6 pods)	30 ± 6 (6 pods)	23 ± 7 (2 pods)	23 ± 4 (2 pods)	23 ± 4 (2 pods)	24 ± 4 (2 pods)
Benzoic acid (µg per puff) Mean of the first 160 puffs	21 ± 3 (2 pods)	41 ± 6 (2 pods)	22 ± 3 (2 pods)				
Acetaldehyde (ng per puff) Mean of the first 160 puffs	76 ± 116 (4 pods)	12 ± 13 (4 pods)					
Acetone (ng per puff) Mean of the first 160 puffs	3 ± 2 (4 pods)	36 ± 10 (4 pods)					
Acrolein (ng per puff) Mean of the first 160 puffs	13 ± 7 (4 pods)	7 ± 2 (4 pods)					
Formaldehyde (ng per puff) Mean of the first 160 puffs	112 ± 117 (4 pods)	11 ± 6 (4 pods)					

For all aerosol measurements, the commercially available mouth piece was used. Contents of benzoic acid in liquids and vapor, and pH values of liquids were determined in lab A. Density, liquid basis composition, nicotine content of liquids, and carbonyl emissions were analyzed in lab B. TPM and nicotine in the emissions were determined in both labs. Values are presented as mean values and corresponding absolute standard deviations

Discussion

Nicotine-salt pod e-cigarettes, especially the market leader JUUL, have started a controversy that first emerged in the US. The combination of factors like product design, viral marketing, and the high nicotine contents in liquids and corresponding aerosols have triggered a great popularity especially among young people, thus raising concerns by US-American authorities (Koh and Douglas 2019; U.S. Surgeon General). In December 2018, JUUL e-cigarettes became available in Europe, where the nicotine contents in the liquids had to be lowered to 20 mg/mL in order to

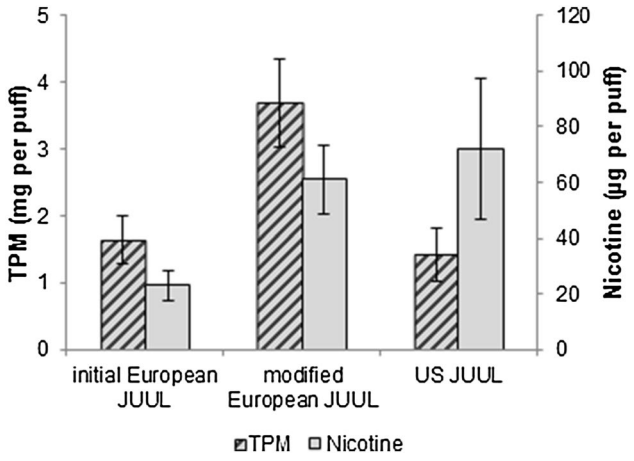

Fig. 2 Total particulate matter (TPM) in mg per puff and nicotine levels in µg per puff released during the first 160 puffs of 20 mg/mL initial European Rich Tobacco JUUL (20 pods), 18 mg/mL modified European Rich Tobacco JUUL (6 pods) and 58 mg/mL US-American Virginia Tobacco JUUL (5 pods)

comply with European regulation (Art. 20 TPD, (European Parliament 2014)). Little is known about the product variants that were placed on the European market.

Our data demonstrate that the initial product on the European market generated a similar amount of vapor when compared to the American version and subsequently only achieved relatively low levels of nicotine delivered into the aerosol. Amidst our investigation, a new product design, referred to as "Turbo", was launched (Mahase 2019). We could show that the degree of vaporization in the newly designed product increased more than twice and therefore can be considered sufficient to compensate for the lower nicotine contents in the liquid. This observation confirmed our initial expectation that nicotine delivery will be increased with technical adaptions. Modified JUUL was shown to deliver approximately the same amounts of nicotine as the American version in our machine vaping set-up and thus could potentially lead to blood nicotine levels that are comparable to tobacco cigarettes as well.

The increased vaporization of modified JUUL is linked to the use of another wick material. It is not related to a higher power delivery as parameters like the resistance of the coil and the battery voltage did not change. The properties of the wick can also have a substantial influence on vaporization via the liquid supply rate. A wick made of an expansible material can resupply the coil faster and more stably with unvaporized e-liquid. It is visible by the naked eye that the constitution of the wick has changed. The initial version of the product showed very high intra- and inter-device deviations, especially when more than 200 puffs were drawn. Vapor generation by modified JUUL is more stable; however the deviations are still high and do not attest a good consistency of nicotine delivery. Since both versions contain the same amount of liquid, only half the number of puffs can be drawn from the modified version (see Supplementary Material). Depending on changes in consumption behavior, this could have an influence on cost.

We also would expect an increased addictiveness of the modified JUUL version due to the higher nicotine delivery. Non-smokers who start vaping e-cigarettes with such a high nicotine delivery per puff are at higher risk to become dependent. If these novel design features were combined with higher power settings, nicotine delivery might increase further. In the case of pod systems, our data possibly support the notion that setting a limit for nicotine delivery into the aerosol (per puff) might be more purposeful than liquid nicotine content limits only. To this end, the nicotine delivery limits should be similar or even lower when compared to tobacco cigarettes. Pod systems are very simple; they do not require any prior knowledge as in the case of conventional e-cigarettes and can be bought and used directly. Setting a general limit of aerosol nicotine levels in pod systems would at least protect initiating adolescents who are getting exposed to these products the first time.

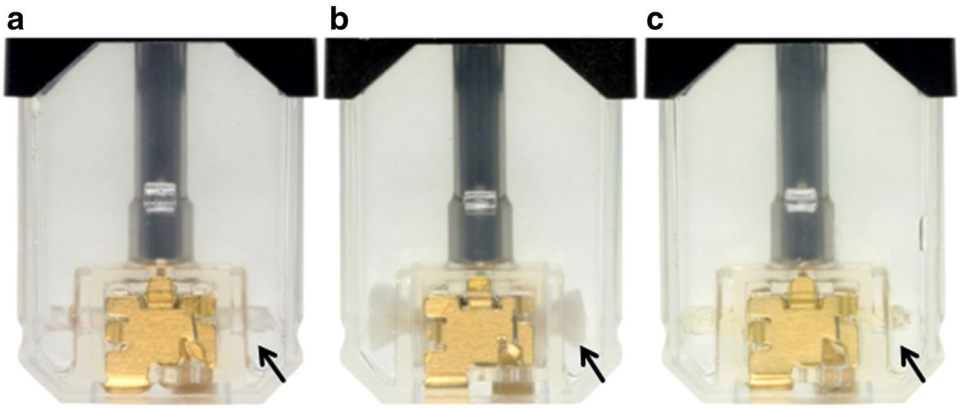

Fig. 3 Photographs of emptied JUUL pods: The initial (**a**) and the American (**c**) variant contain a different wick material than the one used in the modified JUUL version (**b**)

E-cigarettes and e-liquids are complex products that undergo steady product development. Therefore, it can be anticipated that further product innovations will occur and current knowledge becomes quickly outdated. This is of special importance for regulators and surveillance authorities who not only need to keep up with future product development but who are also in charge of monitoring the already existing products. The Sisyphean challenge of tobacco control to keep pace with the development on the market is complicated by practical problems, for example the connectivity of e-cigarette mouth pieces of new shapes to the filter holders of vaping machines used for analytical testing. In the Supplementary Material we demonstrate that suitable adapters can be self-made and lead to comparable results as commercially available options. The adjusted adapters made out of a heat-shrinkable material are cheap and uncomplicated in production and could be considered whenever connection of e-cigarettes to the vaping machine is troublesome and no commercial option is available.

Pod systems are usually operated with low electrical power and therefore provide some advantages from a toxicological perspective. We found levels of carbonyl compounds in the respective aerosols lying in a similar range as reported for the American and non-American JUUL devices (Hiraki et al. 2019; Reilly et al. 2019; Talih et al. 2019), but magnitudes lower than those found in tobacco cigarettes (Counts et al. 2005). Also in relation to other e-cigarettes, especially with higher power settings, the carbonyl emissions by JUUL are still comparatively low (El-Hellani et al. 2018; Talih et al. 2017, 2019).

Tobacco smokers who switch completely to e-cigarettes significantly reduce their levels of exposure to known cigarette toxicants, as shown recently in a 5-days trial by the manufacturer (Jay et al. 2019). A closed system device with a low power setting has the advantage that toxicant generation is comparatively low and no easy manipulation by the consumer is feasible as with open systems. Composition of e-liquids can be regulated and monitored better, although this might be undermined by third-party suppliers of pods and refill solutions.

High nicotine delivery might pose an increased risk for adolescents to initiate nicotine use. On the other side, this feature can be beneficial for smokers who intend to reduce harm or attempt cessation. Satisfying nicotine delivery might suppress urges to smoke and prevent dual use or a relapse to tobacco cigarettes. This has not been achieved by older generations of e-cigarettes (Fearon et al. 2018). But it is yet unclear how these high nicotine levels affect complete cessation, considering that at some point e-cigarette use should be ceased as well. Possible harm reduction is also counteracted by dual use of tobacco and electronic cigarettes that might even increase toxicological health risks for vapers (Osei et al. 2019).

While tobacco smokers, who switch completely to e-cigarettes, can reduce their exposure to known tobacco cigarette toxicants and putatively reduce health risks, non-smokers that start with e-cigarettes jeopardize their health and are prone to develop an addictive disorder. Therefore, initiation of e-cigarette consumption is strongly discouraged for non-smokers irrespective of their age.

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Author contributions NM, HLT, FHS, JH, CH, and AL contributed to the implementation of this e-cigarette research and designed the study. Experiments and data analyses were performed by NM, HLT, MM, SM and AK. NM drafted the manuscript. FHS, CH, PL and AL supervised the study, refined and finished the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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2.1.2. Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans*-3'-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma

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Online Supplementary Material is presented in **Annex II**.

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Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans*-3′-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma

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Phenyl-hexyl column

ABSTRACT

New nicotine delivery products are gaining market share. For evaluation of their characteristics, toxicokinetic investigations are in current research focus. For reliable determination of blood plasma levels of nicotine and its main metabolites cotinine and *trans*-3′-hydroxycotinine, a quantitation method based on LC-ESI-MS/MS was developed and validated. Addition of isotope labeled internal standards prior to rapid sample preparation using protein precipitation with methanol was chosen for sample preparation. Different stationary phases were tested and phenyl-hexyl separation was found to be superior to HILIC, C18, and C8 stationary phases. Ion suppression effects caused by hydrophilic early eluting matrix were eliminated by the adjustment of an adequate retention utilizing a phenyl-hexyl separation stationary phase. Exchange of acetonitrile as organic mobile phase by methanol and elevation of pH value of aqueous mobile phase containing 5 mM NH_4Ac to 4.50 improved the chromatographic resolution. The limits of quantitation for nicotine, cotinine, and hydroxycotinine were 0.15, 0.30, and 0.40 ng/mL, respectively. Linearity was proven by matrix matched calibration for the whole working range from 0.50 ng/mL to 35.0 ng/mL for nicotine and from 6.00 to 420 ng/mL for cotinine and hydroxycotinine (Mandel's fitting test with $R^2 > 0.995$). Quality control samples at four different levels (0.50, 1.50, 17.5, 28.0 ng/mL for nicotine and 6.00, 18.0, 210, 336 ng/mL for cotinine and hydroxycotinine) in plasma were analyzed six times on three days. Mean accuracies ranged from 87.7% to 105.8% for nicotine, from 90.3% to 102.9% for cotinine, and from 99.9% to 109.9% for hydroxycotinine. Intra- and inter-day precisions (RSD %) were below 15% for all analytes (<20% for LLOQ). As proof of concept, the method was successfully applied to a real plasma sample from a cigarette smoking volunteer.

1. Introduction

Blood levels of nicotine after cigarette smoking are an important factor in monitoring of the development and maintenance of nicotine addiction [1–3]. Nicotine replacement therapy (NRT) in smoking cessation is based on the adjustment of a nicotine level in the body that sufficiently suppresses the urge to smoke [3]. Also new products like electronic cigarettes and heated tobacco products are discussed as replacements for combustible cigarettes and as cessation aids [4,5]. However, public health risks like uptake of cigarette smoking by non-smokers are discussed for these products [4–6]. When possible risks and chances of these new products are evaluated, nicotine delivery and toxicokinetics are important factors that need to be studied.

After inhalation, nicotine is rapidly absorbed in the small airways and reaches the brain after 10–20 s. It is widely distributed in the body and undergoes extensive metabolism [1–3]. The most important route of metabolism is mediated via cytochrome P450 (CYP) isoform 2A6 and results in the metabolites cotinine and *trans*-3′-hydroxycotinine (in the following only referred to as hydroxycotinine) as displayed in Fig. 1a [1–3]. The ratio of hydroxycotinine and cotinine is referred to as

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"nicotine metabolic ratio" and is used as an important surrogate marker for CYP 2A6 activity and consequently the status of nicotine metabolism [7–10]. The kinetics of nicotine metabolism is considered to be an important factor for the success of NRT [7]. Studies have shown that slow metabolizers respond better to some types of NRT compared to normal metabolizers. This may be caused by higher nicotine blood levels [10].

Although several validated LC-MS/MS methods for separation of nicotine and metabolites were already published, they did not fit our needs entirely. For example, some groups analyzed nicotine and metabolites using stationary phases with hydrophilic-lipophilic interaction (HILIC) separation principles [11–16]. In all cases, sample preparation includes purification with solid phase extraction (SPE) or liquid–liquid extraction (LLE). Other separation principles like reversed-phase chromatography could be used in combination with a less extensive sample clean-up. Further, phenyl-hexyl based stationary phases combine reverse phase separation with additional retention mechanisms like π-π interactions between the stationary phase and the analytes [17–21]. If analytes contain π-electron systems, for example in aromatic rings, the retention can be enhanced by π-π interactions.

Reliable determination of nicotine and its metabolites cotinine and hydroxycotinine requires a suitable quantitation method that is selective, robust and reproducible. Further, a high sensitivity (LOQ < 0.5 ng/mL nicotine) and quick and easy sample preparation are required for large sample series. To meet all of these criteria, a method based on liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS) applying protein precipitation was developed. The choice of the stationary phase, optimization of chromatography on the phenyl-hexyl stationary phase, the subsequent validation of optimized method, and proof-of-concept application of our developed method on real samples is presented herein.

2. Material and methods

2.1. Chemicals and reagents

All solvents and chemicals were of purity grade suitable for mass spectrometry. (-)-Nicotine (purity ≥ 99%), (±)-nicotine-(methyl-d_3) (isotopic purity ≥ 99%), solutions of (-)-cotinine (purity ≥ 99%), (±)-cotinine-(methyl-d_3) (isotopic purity ≥ 99%), *trans*-3′-hydroxycotinine (purity ≥ 98%; all 1.0 mg/mL in methanol), *trans*-3′-hydroxycotinine-d_3 (isotopic purity ≥ 99%; 100 µg/mL in methanol), and ammonium formate were purchased from Sigma Aldrich (Taufkirchen, Germany). Methanol, acetonitrile, formic acid, and fetal calf serum (FBS superior) were bought from Merck KGaA (Darmstadt, Germany) and ammonium acetate from Honeywell Fluka (Morris Plains, NJ, USA). Nitrogen gas was obtained from Linde (Pullach, Germany) with a purity of 99.999%. Ultrapure water was prepared with a Milli-Q Integral Water Purification System (Merck KGaA, Darmstadt, Germany).

2.2. Human plasma

Human plasma was obtained from healthy volunteers. Blank plasma was donated by non-smokers and tested with the herein described LC-ESI-MS/MS method for impurities prior to use. For proof of concept, venous blood was collected into EDTA monovettes (Sarstedt, Nümbrecht, Germany) from a routine cigarette smoker while smoking a combustible cigarette. The study was approved by the ethics committee of the LMU Munich and performed in accordance with the principles of the Declaration of Helsinki. Full blood was centrifuged for 10 min at 1,500g and 4 °C. To 990 µL sample plasma, 10 µL internal standard mix (see 2.3) was added. Samples were stored at −80 °C and shipped on dry ice.

2.3. Stock solutions, internal standard mix, matrix calibration, and quality control samples

Stock solutions of nicotine, cotinine, and hydroxycotinine were separately prepared in methanol and stored at −20 °C. They were used for the preparation of matrix calibration samples and quality control samples. Stock solutions of internal standards nicotine-d_3, cotinine-d_3, and hydroxycotinine-d_3 were prepared in methanol and stored at −20 °C. A mix was prepared in acetonitrile with a concentration of 500 ng/mL for each internal standard. A reference standard mix (matrix-mix) with 50.0 ng/mL nicotine, 600 ng/mL cotinine, and 600 ng/mL hydroxycotinine was prepared in human blank plasma. Matrix calibration and quality control samples were prepared by spiking different volumes of standard mix into human blank plasma. To 990 µL spiked plasma, 10 µL internal standard mix was added, resulting in a concentration of 5.00 ng/mL per internal standard. Samples were stored at −80 °C. Concentrations of matrix calibration samples and quality control samples are summarized in Table 1.

2.4. Sample preparation

Frozen samples were gently thawed on ice. To 50 µL of plasma, 100 µL of ice-cold methanol was added for protein precipitation. After thorough mixing for 30 s on a vortex shaker (7–2020, neoLab Migge GmbH, Heidelberg, Germany), samples were centrifuged at 4 °C and 14,000 g for 15 min (Centrifuge 5427 R, Eppendorf, Wesseling-Berzdorf,

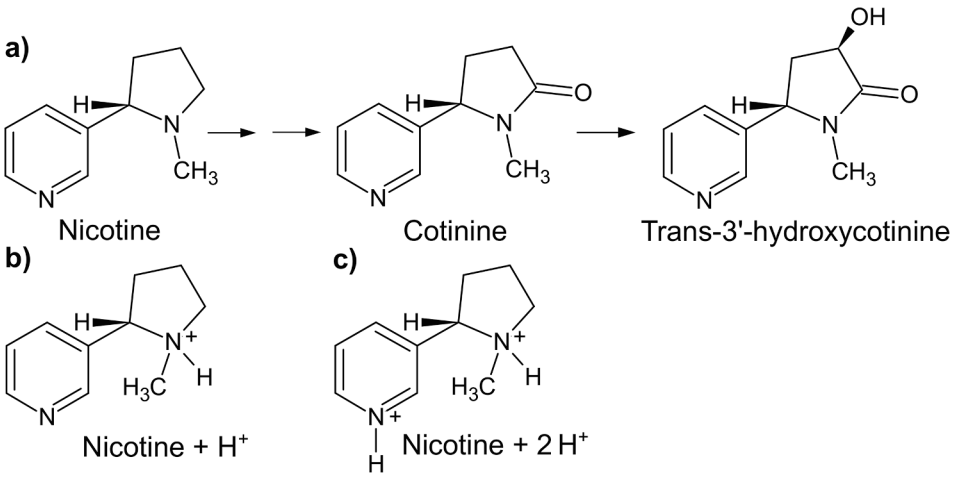

Fig. 1. a) Metabolism of nicotine to its main metabolites cotinine and *trans*-3′-hydroxycotinine. **b)** Protonated nicotine (pyrrolidine nitrogen atom protonated) as dominant form at neutral pH. **c)** Doubly protonated nicotine species (pyridine nitrogen atom protonated as well) as dominated form below pH 3.10.

Table 1
Concentrations of analytes in matrix calibration and quality control samples.

Standard	Nicotine (ng/mL)	Cotinine (ng/mL)	Hydroxycotinine (ng/mL)
Matrix calibration samples			
K1 (LLOQ)	0.50	6.00	6.00
K2	2.50	30.0	30.0
K3	5.00	60.0	60.0
K4	10.0	120	120
K5	15.0	180	180
K6	20.0	240	240
K7	25.0	300	300
K8	30.0	360	360
K9 (ULOQ)	35.0	420	420
Quality control samples			
LLOQ	0.50	6.00	6.00
Low QC (3x LLOQ)	1.50	18.0	18.0
Mid QC (50% ULOQ)	17.5	210	210
High QC (80% ULOQ)	28.0	336	336

Germany). Supernatant was diluted 1:1 with aqueous eluent A to reduce the methanol content for a better separation. The resulting sample solution was used directly for LC-ESI-MS/MS analysis.

2.5. LC-ESI-MS/MS

Analysis was performed with a liquid chromatography system consisting of pumps (LC-20AD), degasser (DGU-20As), auto sampler (SIL-20AC HT), column oven (CTO-20AC), and communications bus module (CBM-20A; all from Prominence series, Shimadzu, Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API4000QTrap, AB Sciex, Framingham, MA, USA) equipped with an electrospray ion source (ESI) operated with Analyst 1.7 software (AB Sciex, Framingham, MA, USA). After injection of 25 µL of final sample solution, separation was achieved on a Luna Phenyl-Hexyl Column (150 mm length, 4.60 mm internal diameter, 3 µm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) equipped with an according guard column (Phenomenex, Torrance, CA, USA) at 45 °C. To prepare eluent A, formic acid was added to 5 mM ammonium acetate in ultrapure water to adjust pH 4.50 ± 0.02 (controlled with 765 Calimatic pH meter; Knick, Berlin, Germany). Methanol was used as eluent B. At a total flow of 1 mL/min, the following gradient was used: Start at 10% B, followed by an increase for 1 min to 30% B with a hold for 1 further min, followed by another increase to 95% B for 2 min and a hold for 2 min, followed by a decrease to 10% for 0.2 min and a hold for 2.8 min. Conditions at the ESI-source were as followed: ion spray voltage, 3800 V; ion source temperature, 650 °C; curtain gas, nitrogen with 10 psi; ion source gas 1, nitrogen with 80 psi; ion source gas 2, nitrogen with 85 psi. Declustering potential was set to 47 V and entrance potential was 7 V. Mass selective detection was performed with multiple reaction monitoring (MRM) mode in positive mode with two transitions per analyte and a detection window of 120 s and a cycle time of 1 s. MRM parameters are summarized in Table 2. In the final method, scheduled multiple reaction monitoring was applied to all transitions. Product ion scan mass spectra of all analytes and internal standards recorded with a collision energy of 52 V are displayed in Figure S.4 (Supplementary Material). Data was analyzed with Software ScieX OS (Version 1.4.0.18067, AB Sciex, Framingham, MA, USA) using the same "Autopeak" integration parameters for all measurements.

2.6. Stationary phase selection

Separation with C18 (EC Nucleosil 100–5 HD C18 column, 150 mm length, 4.60 mm internal diameter, 5 µm particle size, 100 Å pore size; Macherey-Nagel, Düren, Germany) and C8 (EC Nucleosil 120–3 C8 column, 150 mm length, 4.60 mm internal diameter, 3 µm particle size, 120 Å pore size; Macherey-Nagel, Düren, Germany) stationary phases was performed as described under 2.5. In both cases, two mobile phase compositions were tested: 5.00 mM ammonium acetate, 0.1% formic acid in ultra-pure water (eluent A) and the same modifiers in acetonitrile or methanol (eluent B), and also eluent A (5 mM ammonium acetate, formic acid until pH 4.50 in ultra-pure water) and eluent B (methanol) as described under 2.5. Prior to injection, a mix of all standards in methanol (60.0 ng/mL) was diluted 1:2 with the according aqueous eluent A to have the same amount of methanol as in the matrix samples. Separation of matrix samples on C8 and C18 stationary phases has not been tested due to an insufficient separation and bad peak shapes even for matrix-free standards. For this experiment, data acquisition in MRM-mode was not scheduled, but recorded with a fixed dwell time of 70 ms for each transition. Additionally, a HILIC stationary phase (Luna HILIC column, 150 mm length, 3.00 mm internal diameter, 3 µm particle size, 200 Å pore size; with HILIC guard column; Phenomenex, Torrance, CA, USA) was used with an isocratic flow of 5 mM ammonium formate in 95% acetonitrile and 5% ultra-pure water at 0.40 mL/min and 40 °C. Sample preparation prior to HILIC separation was performed as described under 2.4 with the exceptions that proteins were precipitated with 150 µL acetonitrile and that the supernatant was not diluted after centrifugation. At this early stage of method development, fetal calf serum was used as a surrogate matrix, as it is more accessible than nicotine-free human plasma.

Table 2
Parameters for MRM-transitions of quantifiers and qualifiers of analytes and internal standards.

	Q1 mass (Da)	Q3 mass (Da)	Retention time (min)	Collision energy (volts)	Collision exit potential (volts)
Nicotine					
Quantifier	163.2	130.0	3.20	29	6
Qualifier	163.2	132.1	3.20	21	24
Nicotine-d_3					
Quantifier	166.3	132.0	3.20	23	6
Qualifier	166.3	130.0	3.20	45	6
Cotinine					
Quantifier	177.2	98.0	5.20	40	18
Qualifier	177.2	80.0	5.20	25	14
Cotinine-d_3					
Quantifier	180.2	80.0	5.20	35	14
Qualifier	180.2	101.0	5.20	31	18
Hydroxycotinine					
Quantifier	193.1	80.0	4.40	43	14
Qualifier	193.1	134.1	4.40	27	24
Hydroxycotinine-d_3					
Quantifier	196.2	80.0	4.40	41	14
Qualifier	196.2	134.1	4.40	27	24

2.7. Testing of different mobile phases

Eluent A containing 5 mM ammonium acetate in ultra-pure water was adjusted to different pH values by addition of formic acid: pH 2.86 (addition of 0.1% formic acid), pH 3.00, pH 3.50, pH 4.00, pH 4.20, pH 4.30, pH 4.40, pH 4.44, pH 4.50, pH 4.60. Eluent B was prepared with 5 mM ammonium acetate and 0.1% formic acid (and 5% water for acetonitrile) or without modifiers using methanol or acetonitrile. Prior to injection, a mix of all standards in acetonitrile or methanol (60.0 ng/mL) was diluted 1:2 with the according aqueous eluent A to have the same amount of methanol as in the matrix samples. For this experiment, data acquisition in MRM-mode was not scheduled, but recorded with a fixed dwell time of 70 ms for each transition. The Henderson-Hasselbalch equation ($pH = pK_a - \log (C_{acid}/C_{base})$) was used to calculate the proportion of charged analyte molecules [22].

2.8. Characterization of ion suppression

Blank solution (methanol diluted with twofold eluent A) or human blank plasma, prepared as described under 2.4, were analyzed as

described under 2.5. Analyte solution (500 ng/mL in methanol) was infused continuously post-column at a constant flow rate of 20 µL/min using a syringe pump (11 Plus, Harvard Apparatus, March-Hugstetten, Germany) equipped with a 1 mL luer lock syringe (Gastight, Hamilton, Gräfelfing, Germany), while running the analysis of a blank matrix sample. The intensity of the MRM signals for all analytes was monitored over time to characterize ion suppression regions in the chromatogram.

2.9. Characterization of matrix effects

Matrix effects were determined for plasma samples from six different anonymous donors based on EMA Guideline on bioanalytical method validation [23]. Concentrations of Low QC (1.50 ng/mL nicotine, 18.0 ng/mL cotinine, hydroxycotinine) and High QC (28.0 ng/mL nicotine, 336 ng/mL cotinine, hydroxycotinine) were spiked together with internal standard mix (5.00 ng/mL each internal standard in the spiked sample) in the different plasma samples. The same analyte matrix-mix was used as stock to prepare the samples (Low QC: 960 µL plasma sample + 10 µL internal standard mix + 30 µL analyte matrix-mix; High QC: 430 µL plasma sample + 10 µL internal standard mix + 560 µL internal standard mix), because there was not enough nicotine-free plasma available to prepare different analyte matrix-mixes for each sample. Matrix samples were analyzed against matrix-free control samples with the same concentrations. Matrix-free control samples were prepared in methanol and diluted with eluent A to the same ratio of methanol to aqueous part (1:2, v/v) as in the final sample solution. To reach the same concentrations in the final sample, concentrations in methanol prior to dilution were 0.75 ng/mL nicotine, 9.00 ng/mL cotinine and hydroxycotinine, 2.50 ng/mL internal standards for Low QC and 14.0 ng/mL nicotine, 168 ng/mL cotinine and hydroxycotinine, 2.50 ng/mL internal standards for High QC. Internal standard-normalized matrix factors were calculated as described in the Supplementary Material.

2.10. Method validation

Definitions, methods, and criteria for validation were based on international guidelines [23,24]. The criteria that were defined for a successful validation are summarized in Table 3. While most validation experiments were performed according to the current bioanalytical guideline published by EMA [23], additional experiments were performed based on relevant in-house criteria and JRC guideline on methods used in controls of food contact materials [24]. For accuracy and precision, the matrix matched calibration and quality control samples were freshly spiked in pooled matrix at three different days. Matrix calibration samples were prepared (as described under 2.4) and analyzed twice. Quality control samples were prepared and analyzed six times. One of the six resulting sample solutions per quality control level was injected six times in total to assess precision of the instrument. Accuracy was calculated by dividing the found concentrations by the nominal concentrations. Precision was calculated as relative standard deviation. Concentrations resulting in a signal to noise ratio of 3 was defined as limit of detection (LOD) and signal to noise ratio of 10 as limit of quantitation (LOQ). To test for selectivity, six blank plasma samples from different donors were analyzed and checked for interferences for all MRM transitions at the relevant retention times. Assessment of intra-laboratory repeatability was performed additionally to bioanalytical guidelines using different analyte concentrations in the quality control samples (0.75, 12.5, 22.5, 32.5 ng/mL nicotine, 9.00, 150, 270, 390 ng/mL cotinine and hydroxycotinine): Each quality control sample was prepared six times for measurement as described in Section 2.4 to assess precision of sample preparation. One of these quality control samples for each level was injected six times to assess precision of the instrument. The procedure was repeated twice, once by the same person to determine inter-day precision and again by another operator to assess within-laboratory reproducibility.

Table 3
Validation criteria.

Parameter	Criteria
Validation criteria according to bioanalytical guidelines [23]	
Selectivity	No interferences in 6 different matrix samples (response < 20% response of LLOQ for analytes, < 5% for internal standards)
Linearity	Accuracy of at least 75% of calibration samples is 85 – 115% (80 – 120% for LLOQ) with at least 6 calibration points
Accuracy	85 – 115% (80 – 120% for LLOQ)
Precision	≤ 15% (≤20% for LLOQ)
Stability	85 – 115% of nominal value Benchtop: for 5 h on ice and at room temperature Storage: −80 °C for 3 months Freeze and thaw: for at least 3 cycles In autosampler: for 24 h at 15 °C
Matrix factor	CV of ISTD-normalized matrix factors from 6 different matrices ≤ 15%
Additional validation criteria according to other guidelines [24] **and in-house criteria**	
Selectivity	Stability of retention time (±5%); Stable ratio of quantifier and qualifier MRM (±20% deviation);
Linearity	Linear according to Mandel's fitting test and correlation coefficient $R^2 > 0.995$ with at least 6 calibration points covering the whole working range, weighting 1/x
Within-laboratory reproducibility	Difference between accuracies by different operators ≤ 20%

2.11. Stability under benchtop, freeze and thaw, and autosampler conditions

The stability of Low QC (1.50 ng/mL nicotine, 18.0 ng/mL cotinine, hydroxycotinine) and High QC (28.0 ng/mL nicotine, 336 ng/mL cotinine, hydroxycotinine) samples in matrix under defined conditions was determined. For the determination of the benchtop stability, matrix quality control samples were left at room temperature or on ice for up to 5 h. Samples were analyzed in triplicate after 0, 30, 60, 90, 120, 180, 240, and 300 min. Further, stability over 3 freeze and thaw cycles was assessed. Matrix quality control samples were analyzed in triplicate directly after they have been spiked and at three additional days. In between experiments, samples were kept at −80 °C for at least 12 h. They were completely thawed, analyzed in triplicate against a freshly prepared matrix calibration and refrozen at –80 °C. Stability under autosampler conditions was assessed with a triplicate preparation of matrix quality control samples. The resulting samples were divided into 2 vials with 100 µL each. The first set of samples was injected at the beginning of a sequence. The second set was injected at the end of the sequence after 24 h. The closed vials were kept in the autosampler at 15 °C during the 24 h time period.

2.12. Storage stability of frozen samples

The analytes were spiked separately into human plasma containing 5.00 ng/mL internal standard mix. Aliquots were stored at −20 and −80 °C. At days 0, 21, 35, 49, 63, 76, and 119, an aliquot was prepared as described under 2.4 and analyzed. Remaining supernatants were stored at −80 °C until all samples were analyzed again in one run. Recovery was calculated by dividing the measured concentrations after storage by concentrations at day 0 and multiplied with 100%.

3. Results and discussion

3.1. Selection of stationary phase

As a first step, two different separation principles were tested for our analytes: HILIC and reversed-phase separation. Resulting chromatograms are shown in Fig. 2. Using HILIC, injection of matrix-free analytes

in acetonitrile resulted in good separation and acceptable peak-shapes (Fig. 2a). However, when matrix samples were injected, peak shapes, especially of hydroxycotinine, got worse (Fig. 2b). It can be concluded that although HILIC chromatography is well-suited for our analytes, sample preparation protocols that do not remove hydrophilic matrix compounds can lead to significant matrix effects [25]. Since we aimed for a quick and easy sample preparation using only protein precipitation with solvents, the amount of plasma constituents in our samples is supposed to be problematic in combination with HILIC chromatography. Fewer problems are expected with reverse phase chromatography. Thus, a C18 stationary phase has been tested with acetonitrile (Fig. 2c) and with methanol as organic phases. In combination with methanol, chromatograms derived from two exemplary aqueous mobile phases are shown for pH 2.86 (Fig. 2d) and pH 4.50 (Fig. 2e). Separation and peak shapes of cotinine and hydroxycotinine improved when exchanging acetonitrile with methanol and further with increasing pH value. However, the broad peak shape for nicotine improved only slightly from exchanging acetonitrile with methanol, but worsened significantly with the increase of pH value. The same test on a C8 stationary phase (Fig. 2f-h) resulted in a similar observation. Separation of cotinine and hydroxycotinine was acceptable especially with the combination of methanol and pH 4.50. However, the more hydrophobic analyte nicotine did not elute as a defined peak with any of the tested mobile phases. The elution power of acetonitrile and methanol was not sufficient to elute nicotine as a sharp peak from both tested reversed-phase materials. It should be noted that mobile phase gradients were not optimized for C18 and C8 stationary phases. However, alteration of mobile phase gradient was not expected to affect chromatography of nicotine to a satisfactory extent.

Therefore, a phenyl-hexyl stationary phase that combines reverse phase separation with other retention mechanisms like π-π interactions between analytes and stationary phase was selected to improve chromatographic resolution and separation [17–21]. Since optimization of mobile phase plays an important role for separation of our analytes on a phenyl-hexyl column, mobile phase selection is presented under 3.2.

3.2. Selection of mobile phase

Two aspects of the mobile phase have been optimized to achieve good peak shapes especially for the main analyte nicotine: the organic solvent and the pH value. Firstly, acetonitrile in the mobile phase can weaken the influence of π-π interactions on retention [19,21,26,27]. Secondly, nicotine contains two basic nitrogen atoms that may get protonated, resulting in one or two positive charges. The nitrogen atom in the pyrrolidine moiety has basic properties with a pk_a of 8.01 [28]. The second nitrogen atom, located in the aromatic pyridine moiety, has a pk_a of 3.10 and can be protonated under acidic conditions (Fig. 1c) [28]. A positive charge of the pyridine moiety is unfavorable due to negative influence on π-π interactions. Further, to achieve a good peak shape in the chromatograms for the most important analyte nicotine, all nicotine molecules should carry the same charge of + 1. A mixture of differently charged nicotine molecules during chromatography is supposed to cause peak broadening or even double peaks. Thus, the proportion of nicotine molecules with charges at the two basic moieties was calculated using the Henderson-Hasselbalch equation [22]. The results are reported in Table 4. The pyrrolidine moiety is positively charged at all tested pH values. However, protonation of the pyridine ring was below 5% at pH 4.40, leading to acceptable peak shape of nicotine in the chromatogram. To confirm this prediction, actual peak shapes at the different pH values have been compared. Resulting chromatograms are shown in Figs. 3 and 4. For cotinine and hydroxycotinine, the pK_a of the pyridine moiety is expected to be similar to the one for nicotine. However, the introduction of an electronegative carbonyl group into the pyrrolidine moiety leads to a decrease in electron density and a reduction of the basic properties. Thus, the second nitrogen atom is not expected to be protonated at tested pH values.

Further, two different solvents were tested as organic eluent B: acetonitrile and methanol. Results for acetonitrile are shown in Fig. 3: At first, eluent A and B both contained the same modifiers, i.e., 5 mM ammonium acetate and 0.1% formic acid (pH = 2.86 in eluent A, Fig. 3a). Peak splitting for cotinine and peak broadening for nicotine was observed. Then, the pH of the aqueous eluent A was altered, and acetonitrile was used without modifiers (Fig. 3b-d). While cotinine eluted as a single peak, peak splitting was now observed for hydroxycotinine. Chromatography of nicotine did not improve and peak splitting was observed at pH 4.00 (Fig. 3c).

Fig. 4 displays the results obtained using methanol as organic solvent in eluent B. At first, eluent A and B contained the same modifiers (5 mM ammonium acetate and 0.1% formic acid, Fig. 4a). Two peaks for nicotine both with poor retention were observed. Then, pH value of the aqueous eluent A was adjusted and additive-free methanol was used as eluent B (Fig. 4b-i). With increasing pH, peak splitting of nicotine turned into fronting of a single peak at pH 4.00 (Fig. 4c) and 4.20 (Fig. 4d). A further increase resulted in better peak shape and improved retention for nicotine. Peak shape and retention and consequently intensity of cotinine and hydroxycotinine improved as well comparing chromatograms at pH 2.86 (Fig. 4a) and pH 4.50 (Fig. 4h). Chromatographic parameters such as retention time, peak height, full width at half maximum, and tailing factor at different tested pH values with methanol as eluent B are summarized in Table 5. While high values are favorable for the parameters retention time and peak height, full width at half maximum and tailing factors should be low. Chromatographic parameters and especially full width at half maximum were acceptable for all three analytes when determined at pH 4.50 and were sufficiently robust against pH changes. This confirmed that the suitability of the priorly calculated pH value of 4.50 for the aqueous mobile phase.

In addition to the hydrophobic interactions of regular reverse phase columns, phenyl-hexyl columns can achieve additional retention of compounds via π-π interactions. Acetonitrile weakened π-π interactions between analytes and stationary phase. Without the additional binding mechanism, chromatography of cotinine and hydroxycotinine was largely influenced by pH (Fig. 3). The elution order of the three analytes varied depending on the pH value. The use of methanol in contrast resulted in better retention and a different and stable elution order. These observations are in line with existing literature [19,21,27]. Consequently, it can be concluded that π-π interaction as additional retention mechanism improves the chromatography of nicotine, cotinine, and hydroxycotinine. When reversed-phase liquid chromatography is favored, a stationary phase with this additional retention mechanism should be considered and used with methanol and an appropriate pH value. For the final method, a pH value of 4.50 for eluent A (Fig. 4h) was selected. As presented in Table 5, differences in chromatography at pH 4.44 (Fig. 4g) and 4.60 (Fig. 4i) were found to be minor and the quality of the chromatographic separation seems to be robust against small variations in pH value of eluent A. In conclusion, the pH value of mobile phase A is considered as critical control parameter and the use of methanol as organic solvent was found superior compared to acetonitrile.

3.3. Characterization of ion suppression by co-eluting matrix and influence on matrix effects caused by different plasma donors

The aim of our method development was to combine quick sample preparation with high sensitivity, especially for nicotine. Protein precipitation is a quick and very easy sample preparation method, but hardly removes all possible kinds of matrix constituents that can lead to suppression of ionization in certain regions of the chromatogram. When electrospray ionization is used, co-eluting matrix can hamper the ionization of the analytes and result in reduced sensitivity [29–33]. These matrix effects are not limited to the solvent front and may occur due to co-elution of matrix constituents at any time during chromatography [29,31]. To achieve best sensitivity and reproducibility of quantitation,

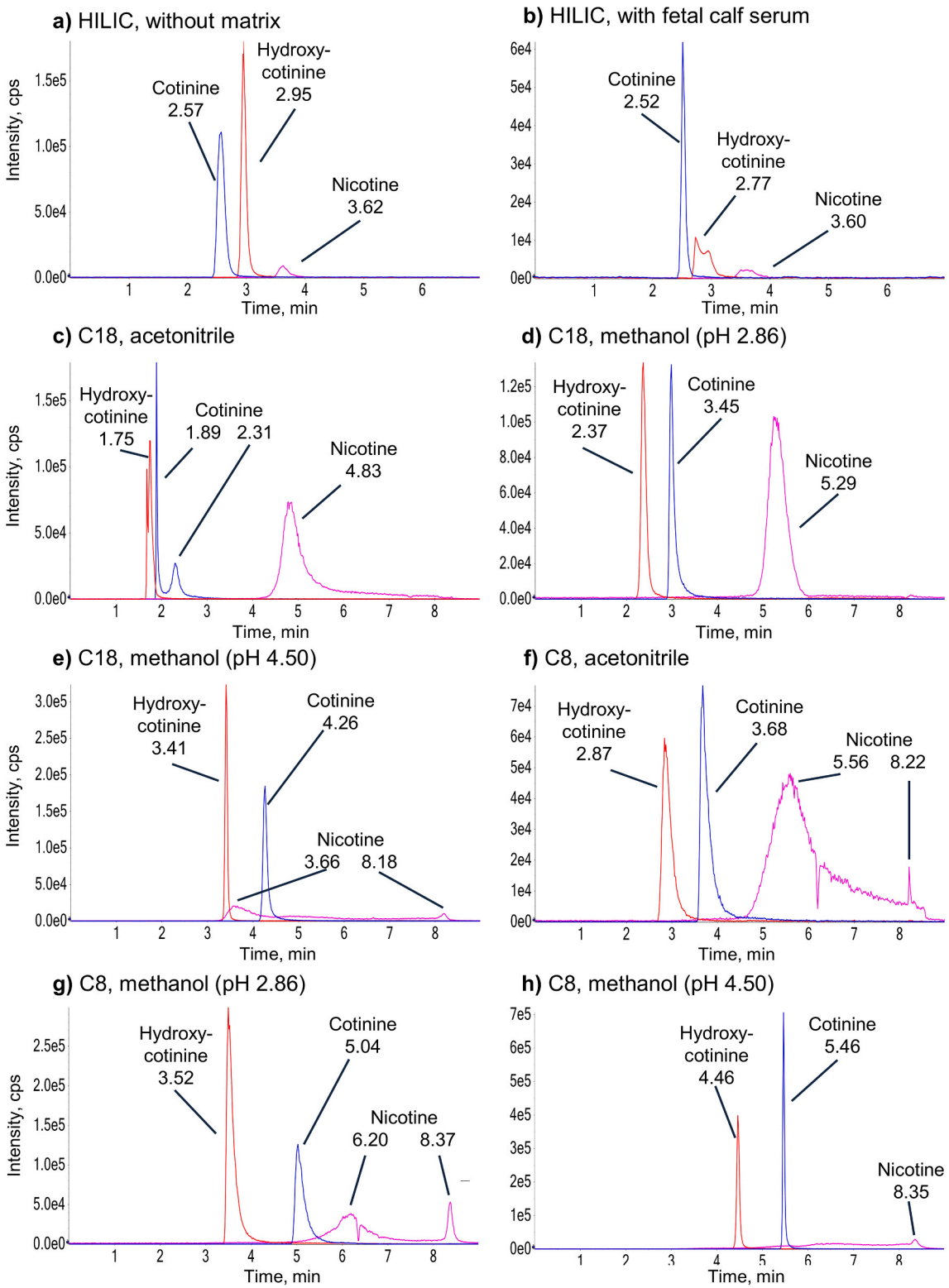

Fig. 2. Chromatograms resulting from different stationary phases: HILIC separation of analytes **a)** in acetonitrile (5.00 ng/mL nicotine, 120 ng/mL cotinine and hydroxycotinine) and **b)** in fetal calf serum after protein precipitation. Separation of analytes without matrix on **c)**, **d)**, and **e)** a C18 stationary phase and **f)**, **g)**, and **h)** a C8 stationary phase. Mobile phases were for **c)** and **f)** 5 mM ammonium acetate, 0.1% formic acid in ultra-pure water (pH 2.86) and the same modifiers in acetonitrile, for **d)** and **g)** 5 mM ammonium acetate, 0.1% formic acid in ultra-pure water (pH 2.86) and the same modifiers in methanol, and for **e)** and **h)** ultra-pure water with 5 mM ammonium acetate and addition of formic acid until pH 4.50 was reached and methanol without modifiers.

Table 4
Proportion of nicotine molecules with positive charge at pyrrolidine and/or at pyridine moiety at different mobile phase pH values.

	Pyrrolidine moiety of nicotine	Pyridine moiety of nicotine
pK_a from [28]	8.01	3.1
pH	% charged	% charged
2.86	100%	63%
3.50	100%	28%
4.00	100%	11%
4.20	100%	7%
4.30	100%	6%
4.40	100%	5%
4.44	100%	4%
4.50	100%	4%
4.60	100%	3%

analytes should not elute at retention times where ion suppression occurs. To test for ion suppression, analyte solution was infused post-column as described under 2.7, based on a procedure suggested by Bonfiglio *et al.* [30]. Intensities of nicotine, cotinine, and hydroxycotinine quantifiers are visualized in Fig. 5. In comparison to matrix-free blank (Fig. 5a), strong ion suppression between 1.5 min and 2 min is observed when matrix is injected (Fig. 5b). The increase of intensity after 5.5 min is due to the high methanol content of 95% in the eluate at that time. High volatility and low surface tension of methanol and the low content of salts like ammonium acetate can improve droplet formation and thus ionization [31]. Since the analytes elute between 3.2 min and 5.2 min, effects of matrix are minor and can be compensated with isotope-labeled internal standards. Although the injected matrix is still complex after the quick sample preparation step, the separation method accomplishes adequate retention of analytes to avoid negative effects due to ion suppression.

To determine differences in matrix effects caused by different plasma donors, internal standard-normalized matrix factors were calculated with nicotine-free venous plasma from six different human donors at two concentrations and are presented in the Supplementary Material (Table S.5). Coefficients of variance (CV) between the six different matrix samples were analyzed per analyte and concentration. CV ranged from 1.3% to 4.9% and were well below the requirement of ≤ 15%.

3.4. Method validation

Calibration curves from day 1 are provided in Figure S.3 for all analytes (Supplementary Material). Results for accuracy and precision tested at 3 days are summarized in Table 6. All criteria from Table 3 that were set prior to validation were fulfilled. Linearity was proven with Mandel's fitting test over the used concentration range with correlation coefficients of the linear regressions (weighted 1/x) higher than 0.999 on day 1. >75% of matrix calibration samples were within ± 15% of the nominal value (±20% for LLOQ) as summarized in the Supplementary Material (Table S.1). Mean accuracies of quality control samples ranged from 87.7% to 109.9%. The precisions of sample preparation and the instrument were below 15% (below 20% for LLOQ) within one day and between three days. As presented in the Supplementary Material (Figure S.1 and Figure S.2), analysis of 6 different blank matrix samples showed no interferences with analytes or internal standards. Mean and standard deviation of retention times were 3.29 ± 0.05 min for nicotine, 3.27 ± 0.05 min for nicotine-d_3, 5.21 ± 0.01 min for cotinine, 5.20 ± 0.01 min for cotinine-d_3, 4.36 ± 0.02 min for hydroxycotinine, and 4.33 ± 0.02 min for hydroxycotinine-d_3, and were the same for quantifier and qualifier MRM. The maximum deviation of ± 5% was not exceeded. Ratios of quantifier and qualifier MRM were found to be within the tolerance of ± 20% for all analytes. Mean and standard deviations of ion ratios were 96.6 ± 7.2% for nicotine, 41.8 ± 6.1% for nicotine-d_3, 352.7 ± 3.4% for cotinine, 34.2 ± 2.4% for cotinine-d_3, 53.4 ± 6.6% for hydroxycotinine, and 50.3 ± 4.2% for hydroxycotinine-d_3. Suitable MRM-transitions for quantifiers and qualifiers were selected during method development and optimized individually for the three analytes

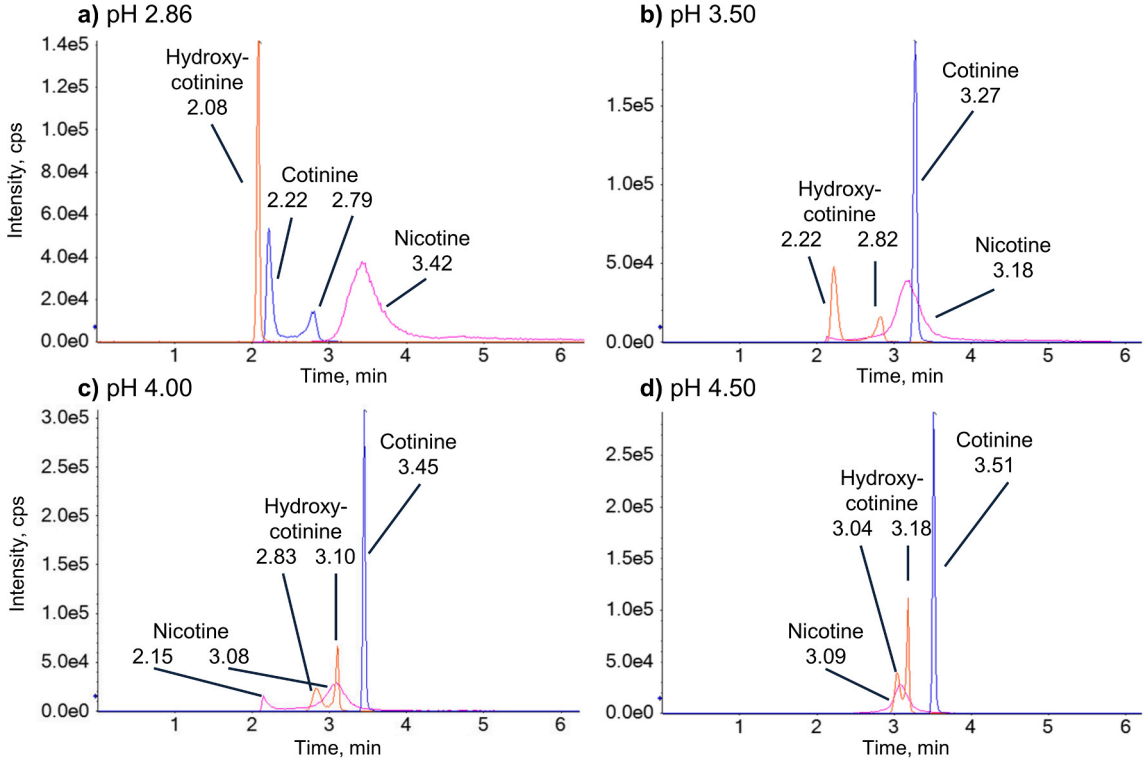

Fig. 3. Chromatograms resulting from different mobile phases: acetonitrile as eluent B containing **a)** 5 mM ammonium acetate, 5% water, and 0.1% formic acid, or **b-d)** without modifiers. Eluent A consisted of 5 mM ammonium acetate in water with **a)** 0.1% formic acid (pH 2.86) or addition of formic acid until **b)** pH 3.50, **c)** pH 4.00, or **d)** pH 4.50 was reached.

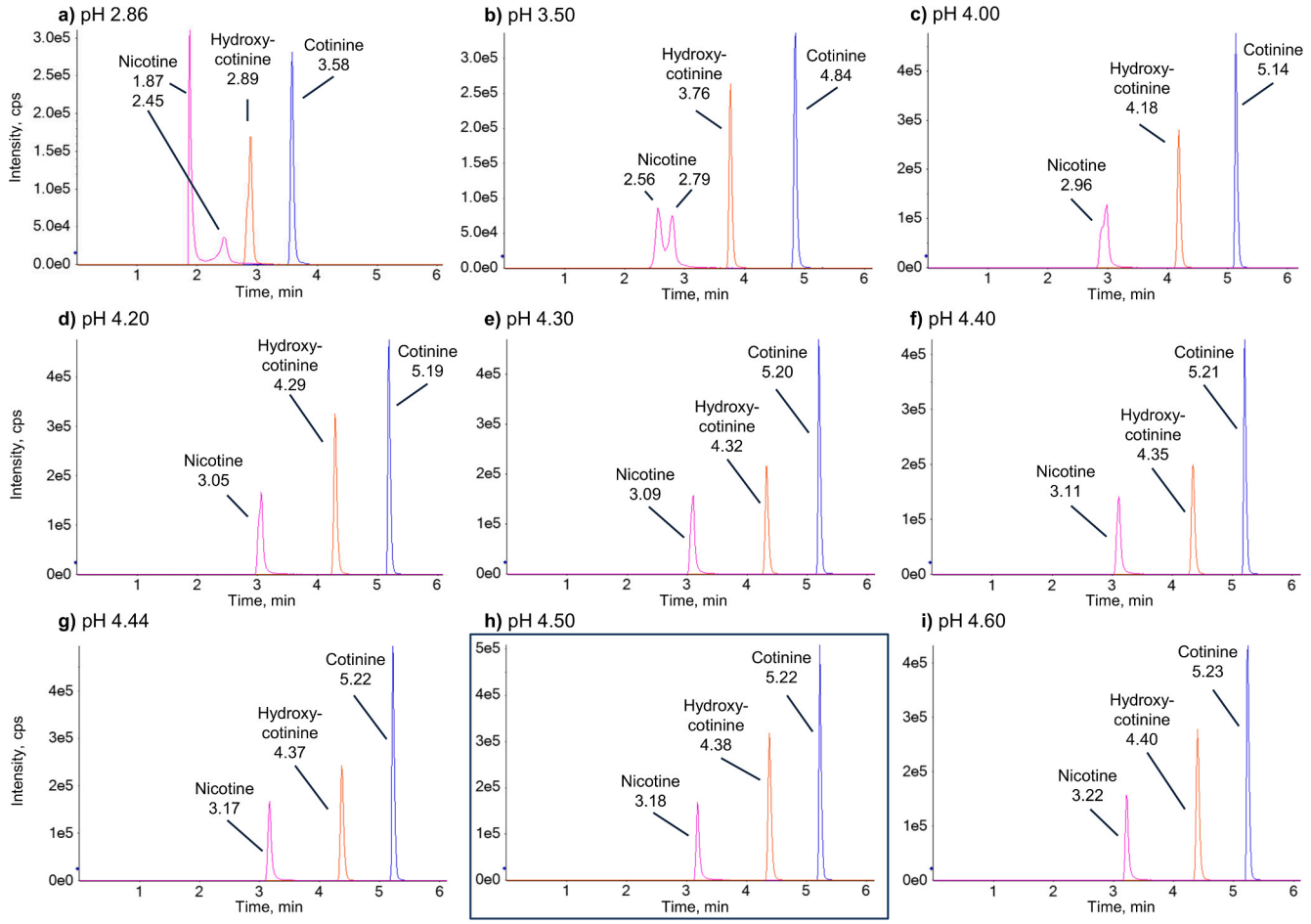

Fig. 4. Chromatograms resulting from different mobile phases: methanol as eluent B containing **a)** 5 mM ammonium acetate and 0.1% formic acid, or **b-i)** without modifiers. Eluent A consisted of 5 mM ammonium acetate in water with **a)** 0.1% formic acid (pH 2.86) or addition of formic acid until **b)** pH 3.50, **c)** pH 4.00, **d)** pH 4.20, **e)** pH 4.30, **f)** pH 4.40, **g)** pH 4.44, **h)** pH 4.50, or **i)** pH 4.60 was reached.

Table 5
Retention time (RT, in min), peak height (in cps), full width at half maximum (FWHM, in min), and tailing factor (Tf) for all analytes at different pH values of eluent A with methanol as eluent B.

pH	2.86	3.5	4	4.2	4.3	4.4	4.44	4.5	4.6
Nicotine									
RT	1.9	2.7	3.0	3.1	3.1	3.1	3.2	3.2	3.2
Height	$3.1x10^5$	$8.6x10^4$	$1.2x10^5$	$1.5x10^5$	$1.5x10^5$	$1.3x10^5$	$1.5x10^5$	$1.4x10^5$	$1.3x10^5$
FWHM	0.04	0.32	0.08	0.09	0.08	0.08	0.07	0.06	0.06
Tf	11.51	2.64	0.86	1.08	1.24	1.30	1.32	1.52	1.64
Cotinine									
RT	3.6	4.8	5.1	5.2	5.2	5.2	5.2	5.2	5.2
Height	$2.8x10^5$	$3.4x10^5$	$4.8x10^5$	$4.8x10^5$	$4.7x10^5$	$4.3x10^5$	$4.9x10^5$	$5.1x10^5$	$4.3x10^5$
FWHM	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Tf	1.14	1.22	1.78	1.32	1.78	1.64	1.50	1.61	1.24
Hydroxycotinine									
RT	2.9	3.8	4.2	4.3	4.3	4.4	4.4	4.4	4.4
Height	$1.7x10^5$	$2.6x10^5$	$2.8x10^5$	$3.2x10^5$	$2.2x10^5$	$2.0x10^5$	$2.4x10^5$	$3.2x10^5$	$2.8x10^5$
FWHM	0.07	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.06
Tf	0.98	1.14	1.19	1.39	1.37	1.19	1.44	1.31	1.35

with regard to signal-to-noise ratios and linearity of the working range. For cotinine, the MRM-transition with the highest intensity was used as qualifier instead of quantifier to obtain the best possible linear fit over the whole working range. Standard deviations of ion rations were highest when low concentrations of nicotine and high concentrations of cotinine were analyzed since they were close to lower or upper end of the linear range. Results for intra-laboratory repeatability that was assessed additional to bioanalytical guidelines using other concentrations of quality control samples (0.75, 12.5, 22.5, 32.5 ng/mL nicotine, 9.00, 150, 270, 390 ng/mL cotinine and hydroxycotinine) are presented in the Supplementary Material (Table S.6). Deviation of accuracies between operators was below 20%. The method was repeatable and reproducible within the laboratory. Estimated LOD and LOQ are shown in Table 7. The required sensitivity has been achieved.

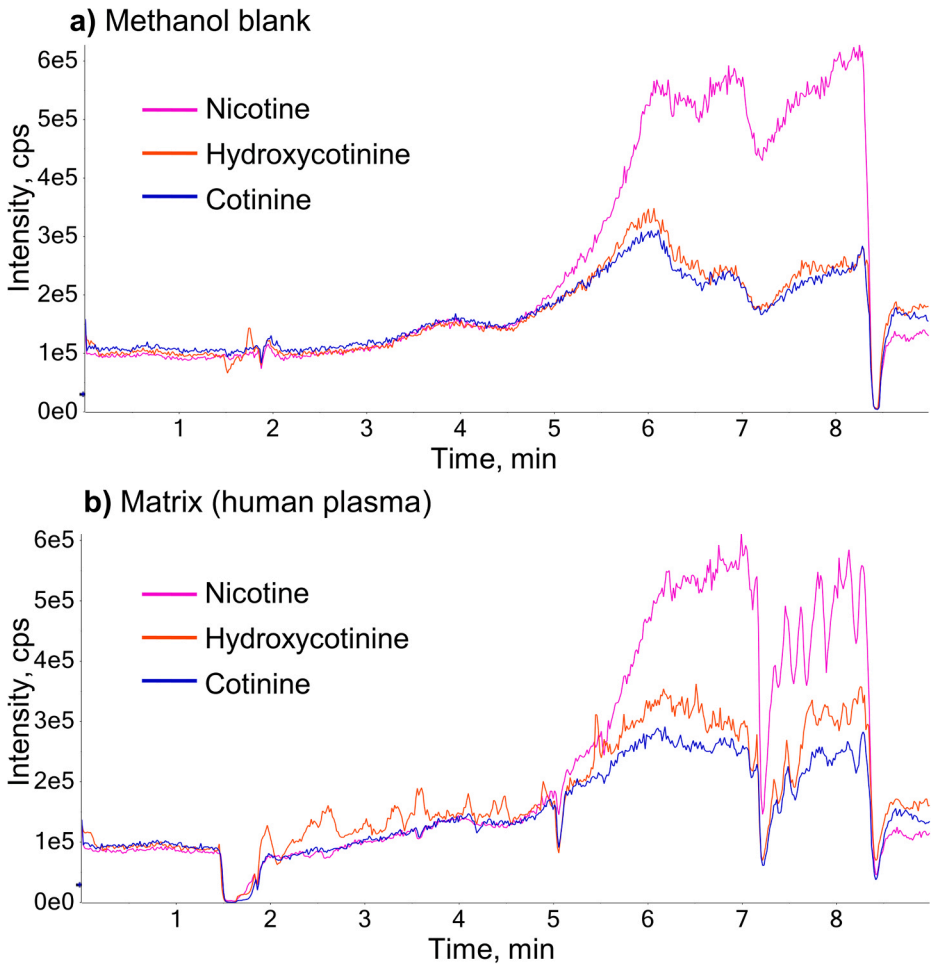

Fig. 5. Influence of eluting **a)** blank or **b)** matrix on intensities of post-column infused analytes.

Table 6
Overview of validation results: Accuracy and precision.

Concentration ng/mL	Day 1 Accuracy (%)	Precision (%)	Precision of injection (%)	Day 2 Accuracy (%)	Precision (%)	Day 3 Accuracy (%)	Precision (%)	Intra-day precision (%)
Nicotine								
0.50 (LLOQ)	105.8	9.3	9.6	87.7	7.7	96.0	16.3	13.6
1.50	98.5	6.0	5.5	89.8	5.0	97.4	6.7	7.0
17.5	97.1	1.2	1.4	100.8	6.6	95.1	4.0	4.9
28.0	96.0	1.9	2.0	98.2	1.9	96.3	2.3	2.2
Cotinine								
6.00 (LLOQ)	102.9	4.8	4.8	90.3	5.5	90.7	9.1	8.9
18.0	100.4	1.8	1.5	91.8	3.6	98.6	3.8	4.9
210	94.1	1.4	1.6	94.8	1.9	97.3	2.5	2.4
336	97.1	1.2	1.0	95.8	1.5	99.0	1.7	2.0
Hydroxycotinine								
6.00 (LLOQ)	103.4	5.7	3.9	109.9	4.5	103.5	12.6	8.3
18.0	102.0	5.7	3.5	101.9	6.6	109.1	2.8	5.9
210	99.9	3.3	2.5	103.1	1.2	105.6	5.1	4.1
336	100.0	3.3	3.8	106.1	1.6	105.0	2.9	3.6

Table 7
Limits of detection (LOD) and limits of quantitation (LOQ) for all analytes.

Analyte	LOD (ng/mL)	LOQ (ng/mL)
Nicotine	0.05	0.15
Cotinine	0.09	0.30
Hydroxycotinine	0.12	0.40

3.5. Stability under benchtop, freeze and thaw, and autosampler conditions and long-term storage stability in human plasma

Concentrations of analytes was within 85% – 115% of the nominal value for both QC levels under the tested conditions. Recoveries after short-term storage on ice ranged from 91.6% to 109.6% and at room temperature from 86.2% to 111.4% after 5 h. After the third freeze and thaw cycle, the recovery ranged from 94.6% to 108.2%. Recoveries of samples that were kept under autosampler conditions at 15 °C for 24 h

ranged from 99.2% to 113.8%. Further details and the complete data set are presented in the Supplementary Material (Tables S.2 – S.4).

As plotted in Fig. 6, all analytes remained stable over 119 days for both storage conditions, −80 °C and −20 °C. No increase of cotinine and hydroxycotinine was found over time, indicating that no further metabolism of nicotine took place during storing time. Thus, samples that have been already spiked with internal standards can be stored for at least 119 days at either −80 °C or −20 °C. Additionally, stability was assessed after 344 days of storage at −80 °C. Recoveries for nicotine, cotinine, and hydroxycotinine ranged from 94.7% to 102.9%, confirming stability for the extended time period. Concentrations of analytes were assessed directly at sampling day and again in one run at the last sampling day. The concentrations of supernatants that were sampled at day 0 did not decrease after storage at −80 °C for 119 days. This shows that supernatants after protein precipitation can be stored at −80 °C for at least 119 days prior to dilution with eluent A.

3.6. Example chromatograms and application to real sample

Nicotine and its metabolites cotinine and hydroxycotinine were quantified. Representative chromatograms derived from human blank matrix with and without addition of internal standards and from a matrix calibration sample are shown in Fig. 7. For reasons of clarity, analytes and internal standards are presented in separate parts. Human blank plasma was free of peaks for analytes and internal standards (Fig. 7 a and b). Blank plasma spiked with 5.00 ng/mL internal standards was found to be free of analyte peaks (Fig. 7 c and d). Spiking of analytes and internal standards to human blank plasma (5.00 ng/mL nicotine, 60.0 ng/mL cotinine, 60.0 ng/mL hydroxycotinine, 5.00 ng/mL internal standards) resulted in the chromatogram shown in Fig. 7 g and h. The chromatogram at LLOQ (0.50 ng/mL nicotine, 6.00 ng/mL cotinine, 6.00 ng/mL hydroxycotinine, 5.00 ng/mL internal standards) is shown in Fig. 7 e and f. Signal to noise ratio of nicotine was 16.6. For proof of concept, the method was applied to real plasma samples that were taken from a volunteer (male, 30 years old) during a smoking session of a combustible cigarette. A routine cigarette smoker drew 2 puffs per minute from a conventional cigarette for 5 min. Blood was collected before and at different time points during and after the smoking session. It was processed as described in Sections 2.2 and 2.4. Fig. 7i and j show chromatograms resulting from real plasma, sampled 8 min after the volunteer started the procedure. The analytes nicotine, cotinine, and hydroxycotinine were quantified as 7.94 ng/mL, 61.2 ng/mL, and 32.2 ng/mL, respectively. Quantitation of the metabolites cotinine and

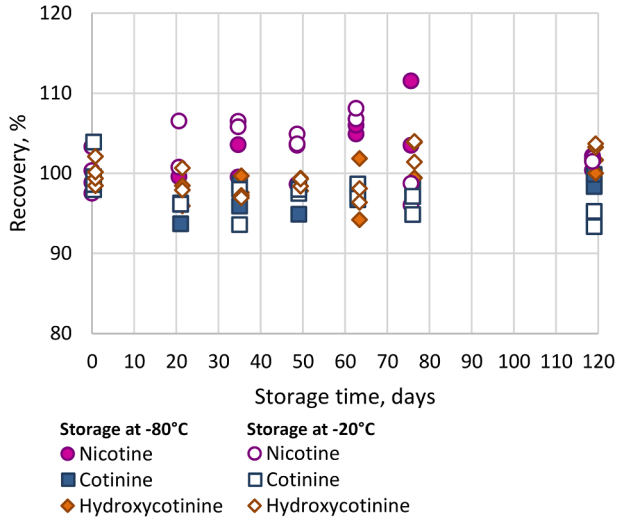

Fig. 6. Recovery of analytes after storage at −80 or −20 °C measured in one run at day 119.

hydroxycotinine plays an important role in addition to the determination of nicotine. Their ratio, calculated by dividing the plasma concentration of hydroxycotinine by the plasma concentration of cotinine, can be used as a surrogate marker for CYP 2A6 metabolic activity which is the main enzyme for nicotine metabolism [7–10]. Previous studies have shown that rate of nicotine metabolism is a factor for success of some NRT, likely due to higher nicotine blood levels [7–10]. Slow metabolizers were found to have lower nicotine metabolic ratios compared to normal metabolizers. A cut-off level of < 0.31 for slow metabolizers and > 0.31 for normal metabolizers has been described in the literature based on their data set of 1246 participants [9]. The nicotine metabolic ratio derived from the test smoker in this real plasma sample was 0.53, above the exemplary cut-off value of 0.31. Accordingly, the test person was classified as a normal metabolizer. Information on nicotine metabolic ratio should be assessed in parallel to nicotine plasma concentrations since it provides additional information on the metabolic status without additional testing. Nicotine metabolism can potentially influence consumption pattern or nicotine kinetics of the studied product. Thus, an analytical method that is developed for determination of nicotine in plasma should ideally include the analytes cotinine and hydroxycotinine as well.

3.7. Advantages of the method

The aim of this method development was to achieve high sensitivity for nicotine (LOQ < 0.5 ng/mL) without a time-consuming sample preparation procedure. Other well-documented methods for the determination of nicotine and/or its metabolites from biological matrices like blood plasma and urine include elaborate sample preparation protocols that are more complicated and time-consuming like solid-phase extraction [12,13,15,34–38] or liquid–liquid extraction [11,14,39–41] or even both [16]. However, sample handling time is only one aspect of many. The LOQ and the lowest level of the linear working range have to be suitable for the intended application. The main purpose of the method described herein is to quantify the rise in nicotine blood levels during use of nicotine delivery products in consumers. Since the volunteers will be asked to be abstinent from nicotine consumption overnight, blood level at t0 (directly prior to administration) are expected to be very low. Thus, the lowest level of the linear working range of nicotine should be 0.5 ng/mL. Some of the previously mentioned methods with a time-consuming sample clean-up step reported a LOQ for nicotine of 1 ng/mL or lower [11,12,15,16,35,37,41]. Yuan *et al.* performed protein precipitation and removed remaining matrix with online turbulent flow extraction prior to separation and reported a LOQ of below 0.5 ng/mL [42]. Another validated method combined protein precipitation with reverse phase chromatography, reporting a LOQ of 3 ng/mL [43]. The required high sensitivity for nicotine despite the high amount of remaining matrix constituents in the samples has been achieved due to prolonged retention of analytes in the herein described method. The first analyte nicotine elutes at 3.2 min while strong ion suppression due to matrix constituents has been present between 1.5 and 2 min. This extension of the retention time of nicotine to more than double than the solvent front time was accomplished by an increase of eluent pH value. At pH 4.50, the nitrogen atom in the pyridine ring of nicotine is predominantly uncharged leading to an enhanced interaction with the stationary phase. A further advantage of the herein described method is the low requirement for laboratory equipment. No special apparatus is needed for sample clean-up and the method runs stably on an older generation mass spectrometer (4000er series). If required for the study, sensitivity can possibly be increased further by switching to a newer generation mass spectrometer.

4. Conclusion

Protein precipitation is a very simple and rapid sample preparation technique with a minimum amount of sample handling time as well as

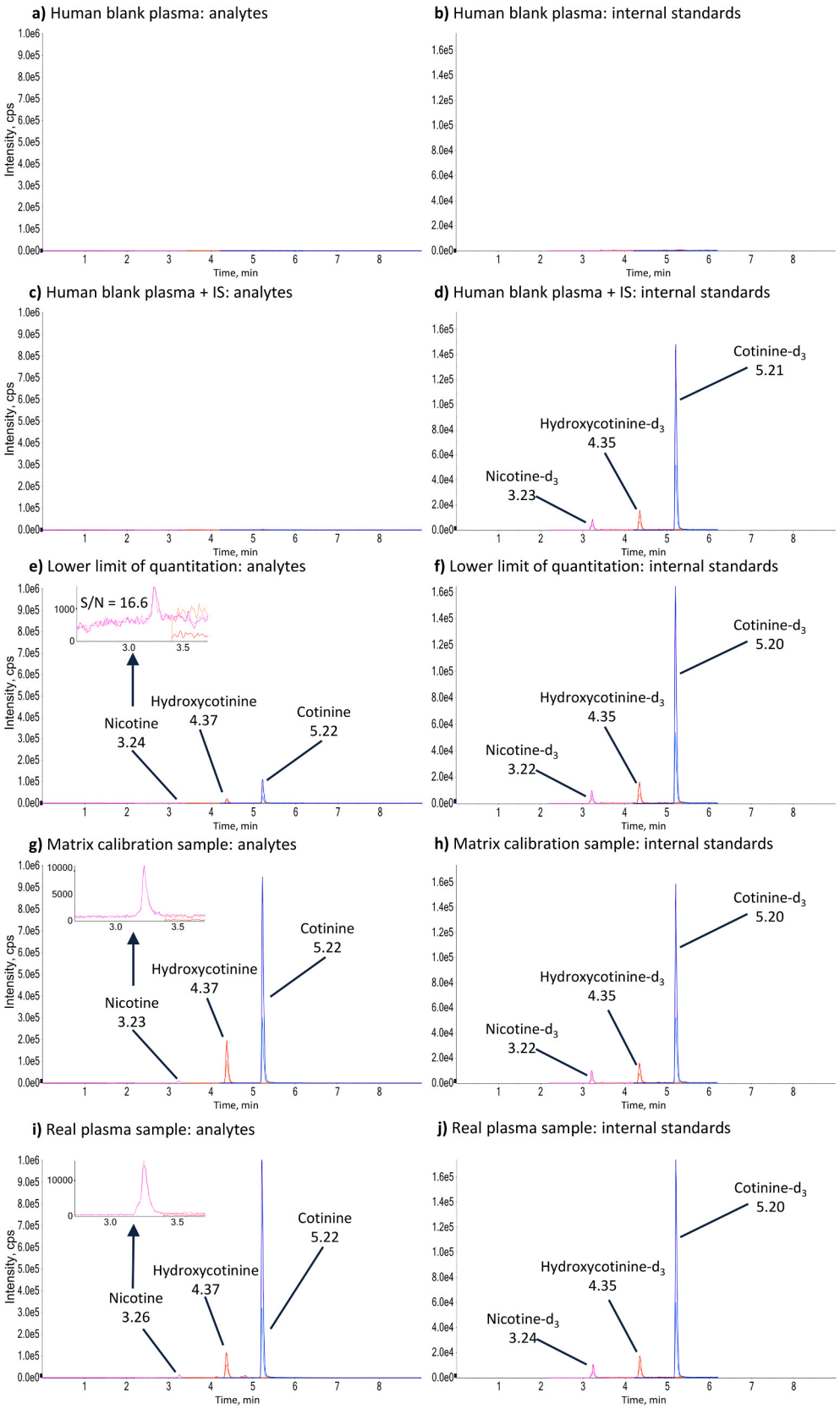

Fig. 7. Example chromatograms with quantifier and qualifier traces of analytes (a, c, e, g, i) and internal standards (b, d, f, h, j) in **a)** and **b)** human blank plasma, **c)** and **d)** human blank plasma spiked with 5.00 ng/mL internal standards, **e)** and **f)** LLOQ (0.50 ng/mL nicotine, 6.0 ng/mL cotinine and hydroxycotinine, 5.00 ng/mL internal standards), integrated zoom for the nicotine signal (signal to noise ratio 16.6), **g)** and **h)** matrix calibration sample (5.00 ng/mL nicotine, 60.0 ng/mL cotinine and hydroxycotinine, 5.00 ng/mL internal standards), integrated zoom for the nicotine signal, **i)** and **j)** real plasma sample from a smoking volunteer, integrated zoom for the nicotine signal. For all analytes but cotinine and all internal standards, the quantifier trace shows the higher signal.

sample amount needed. Human plasma was mixed with cold methanol to precipitate proteins. After centrifugation, the supernatant was diluted with aqueous eluent A to reduce the amount of methanol prior to injection into the LC-ESI-MS/MS system. This fast, robust, and sensitive procedure allows a high throughput of samples. Remaining matrix after protein precipitation can potentially interfere with the ionization of co-eluting analytes and thus reduce sensitivity. Improved retention of analytes can separate elution and ionization of analytes from early

eluting matrix components and consequently reduce ion suppression. Thus, a phenyl-hexyl stationary phase was selected, and mobile phase composition was optimized to improve π-π interactions between stationary phase and analytes. A pH value of 4.50 was selected for aqueous eluent A to avoid protonation of the pyridine ring of nicotine. As organic eluent B, methanol was shown to be superior to acetonitrile. Ion suppression of co-eluting matrix components was assessed with a post-column infusion setup and confirmed to be low. The resulting LC-ESI-MS/MS method for quantitation of nicotine and its most important metabolites cotinine and hydroxycotinine in human plasma was validated with a linear working range of 0.50–35.0 ng/mL for nicotine and 6.00 to 420 ng/mL for cotinine and hydroxycotinine. The method was shown to be selective, sensitive, reproducible, repeatable, and rapid with an easy sample preparation step. Application to real plasma samples of a smoking volunteer was successful. The herein described protocol will be used in an ongoing study on nicotine delivery by electronic cigarettes and may be adopted by other laboratories with similar projects.

CRediT authorship contribution statement

Nadja Mallock: Conceptualization, Investigation, Validation, Writing - original draft, Methodology. **Andrea Rabenstein:** Conceptualization. **Peter Laux:** Conceptualization, Writing - review & editing. **Tobias Rüther:** Conceptualization. **Christoph Hutzler:** Supervision, Conceptualization, Writing - review & editing, Validation. **Maria Kristina Parr:** Supervision, Conceptualization, Writing - review & editing. **Andreas Luch:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122736.

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2.1.3. Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after pod modification

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Online Supplementary Material is presented in Annex III.

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Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after pod modification

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The emergence of e-cigarettes on the consumer market led to a tremendous rise in e-cigarette consumption among adolescents in the United States. The success of JUUL and other pod systems was linked to its high nicotine delivery capacity. In compliance with the European Tobacco Product directive, liquid nicotine contents in the European JUUL variants are limited to 20 mg/mL or below. A short time after launching the initial version in Europe, JUUL pods have been modified in terms of the wick material used. This modification has been demonstrated previously to lead to an elevated aerosol generation, consequently, to a larger amount of nicotine per puff generated. The present study was designed to assess whether the mentioned differences between the "initial" and "modified" JUUL versions may cause a significant difference during consumption, and how nicotine delivery compares with tobacco cigarettes. In this single-center three-arm study, nicotine pharmacokinetics and influence on urge to smoke/vape were compared for tobacco cigarettes, the "initial" version of the European JUUL, and the "modified" version of the European JUUL. Participants, 15 active smokers and 17 active e-cigarette users, were instructed to consume their study product according to a pre-directed puffing protocol. Venous blood was sampled for nicotine analysis to cover the acute phase and the first 30 min after starting. Nicotine delivery and the reduction of urge to smoke/vape upon usage of both European JUUL variants were lower in comparison to tobacco cigarettes. This suggests a lower addictive potential. Modification of the pod design did not result in significant differences at the first ten puffs, as confirmed by a vaping machine experiment. Apparently, the limitations by the initially used wick material only come into effect after longer usage time.

Tobacco smoking is a major avoidable health risk, accounting for more than 8 million premature fatalities every year including second hand exposure[1]. The World Health Organization has recognized tobacco consumption as "global epidemic" that must be counteracted by intensified efforts of tobacco control[2]. Smoking cessation is difficult as nicotine is a strong incentive for continued smoking leading to addiction[3]. Diseases induced by cigarette smoking are predominantly linked to hazardous constituents and combustion products, such as the carcinogens benzo[*a*]pyrene, 1,3-butadiene, and formaldehyde[4]. In recent years, a wide range of nicotine delivery devices has entered the market. E-cigarettes aerosolize liquids consisting of propylene glycol and glycerol and optionally containing nicotine and different aroma compounds. Liquids can also contain toxicologically relevant ingredients[5,6] or impurities as for example tobacco-specific nitrosamines[7,8]. Further, hazardous substances like carbonyl compounds[6] or flavorant-solvent adducts[9,10] can be formed during heating. Typically, levels of tobacco related toxicants are strongly reduced in the aerosol as compared with cigarette smoke[11]. Consequently, exclusive use of vaping products facilitates a significantly reduced toxicant exposure in comparison to smoking including dual use of cigarettes and e-cigarettes[12]. Some harm reduction strategies encourage a complete switch

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to e-cigarettes for smokers who are unable to quit cigarette smoking[13]. Putative health benefits are not anticipated for dual users who consume e-cigarettes and conventional cigarettes in parallel. An increased exposure to tobacco toxicants has even been reported for dual users, as compared with exclusive smokers of conventional cigarettes[12]. To support a complete refrain from combustible cigarettes, nicotine delivery by e-cigarettes needs to be sufficiently high to provide an alternative[14]. Importantly, e-cigarettes with high nicotine delivery are addictive and discussed for non-smokers as a gateway to smoking[15,16]. Whereas early e-cigarettes showed only a limited capacity to reach nicotine blood levels in the range of combustible cigarettes, this has been achieved by newer product generations[17,18]. The US American version of the pod e-cigarette JUUL has a low vaporization power but contains high nicotine concentrations of up to 5% (59 mg/mL) in the liquid[19]. Nicotine delivery in previous studies was comparable to combustible cigarettes in experienced e-cigarette users[20] but lower in e-cigarette naïve participants[21,22]. This shows that the American version of JUUL has the potential for a high nicotine delivery that is dependent on inhalation and user's experience with e-cigarettes. The number of adolescent vapers has risen in the recent years, causing a "public health epidemic", as stated by the US Surgeon General[23]. Signs of nicotine dependence have been reported for adolescent users of pod e-cigarettes[24]. In a recent US study, 13.5% of adolescents and young adults were ever users of the e-cigarette brand JUUL, 6.1% were current users[25]. Reasons for the high acceptance and attractiveness of this e-cigarette brand presumably include curiosity, appealing smell due to the used flavors, convenience of use, initial marketing that was targeting young people, an inconspicuous product design that has led to internet challenges, product use by peers, as well as a high nicotine delivery[26–29].

JUUL sales in Europe have started end of 2018 with a rather low-key marketing. A comparable impact on nicotine consumption by young people has not been reported for Europe. The big market success as seen in the US failed to repeat in Europe. Furthermore, JUUL labs have announced a withdrawal of JUUL e-cigarettes from some European countries such as Austria, Germany, and Switzerland[30]. The European Tobacco Product Directive (TPD) limits nicotine content in e-liquids to 20 mg/mL[31]. Accordingly, the European version of the product contains only 18 mg/mL nicotine in the liquid, approximately a third of the concentration in the high nicotine US version. Initially, the nicotine level in emissions generated with a vaping machine using a puffing regimen for e-cigarettes (CRM 81) was three times lower when compared to the US version[32]. After a technical modification of the wick material inside the pods that provides liquid to the coil, vapor generation of the EU version increased and nicotine content per puff approximated the US version[32]. A recent human consumption study showed a lower nicotine delivery for the European JUUL version[33], but did not analyze whether this can be improved by the technical modifications of the different European products. However, data on the nicotine delivery by different versions of JUUL can provide general insight into the capacity and limitations of modern low powered e-cigarettes and is required for their risk assessment. We have assessed parameters such as nicotine pharmacokinetics, urge to smoke, and adverse effects after consumption of JUUL e-cigarettes and conventional cigarettes for experienced e-cigarette users and cigarette smokers using a pre-directed puffing regimen.

Methods

Aim and ethics. Aim of the study was to get information about the addictive potential and addiction satisfaction of the European JUUL ("initial" and "modified" versions) compared to a tobacco cigarette. Therefore, we analyzed nicotine delivery of these products, especially in the acute phase, by examining venous blood plasma. The study was approved by the ethics committee of the LMU Munich (Amendment to project number 72-15) and performed in accordance with the principles of the Declaration of Helsinki in the currently valid version. It was registered at the DRKS (DRKS00017432). Informed consent was obtained from all participants before participation in the study.

Study products and groups. The study was designed as a single-center three-arm study. The products we used were (a) commercial, combustible tobacco cigarettes (Marlboro Red, Philip & Morris), (b) JUUL e-cigarettes with the new technology (JUUL "modified") with rich tobacco flavor, and (c) JUUL e-cigarettes with the old technology (JUUL "initial") with rich tobacco flavor. As previously published, the wick used in "modified" JUUL pods consist of a different material than the wick used in the "initial" JUUL pods[32]. Participants received the same instruction for use of both JUUL variants according to the producer manual. Products were purchased in local stores in Berlin and Munich, Germany, and online.

Participants. This single-center three-arm study included 15 active smokers and 17 active e-cigarette users who were tested with one or both products. 15 sessions were performed for cigarettes, 15 for the modified JUUL, and 11 for the initial JUUL version. This gives a total of 41 experimental sessions. Data from one participant that did not show any increase in nicotine plasma concentration were excluded from analysis. The participants were divided into either the tobacco cigarette group or one of the e-cigarette groups according to the product they normally used. The participants were recruited for participation in the study via advertisement with flyers and the internet. Participants were enrolled in the study after inclusion and exclusion criteria had been checked and participants had provided written informed consent. Inclusion criteria for all volunteers: Age between 18 and 55 years, 12 h of abstinence (e-cigarette and tobacco cigarette consumption), CO levels < 5 ppm (measurement in the expiratory air using a micro-smokerlyzer; Bedfont Scientific Ltd., Anif, Austria) to verify smoking abstinence, and ability to give consent. Special inclusion criteria for electronic cigarette users were e-cigarette use for > 3 months, daily consumption, no daily consumption of conventional tobacco cigarettes for > 3 months. Special inclusion criteria for tobacco cigarette smokers were daily smoking for > 5 years, consumption of > 10 cigarettes/day.

Exclusion criteria for both (electronic cigarette users and tobacco cigarette smokers): Participants under 18 or over 55 years of age, acute psychiatric illness according to ICD-10/DSM IV, other serious psychiatric disorders,

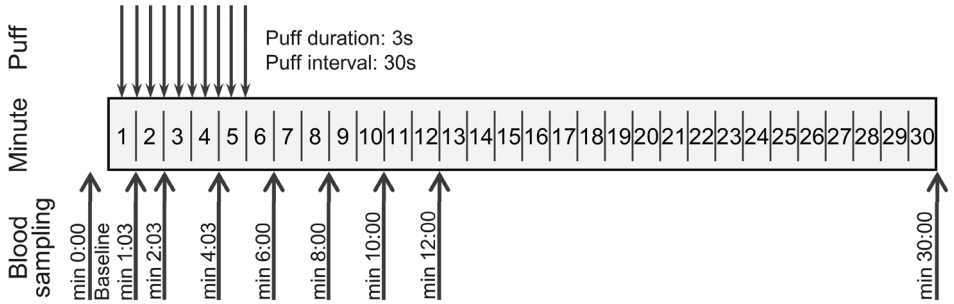

Figure 1. Study design.

acute suicidality, existing pregnancy, breastfeeding, drug, medication, or alcohol abuse at the time of the study, malignant cancer in the past 5 years, serious internal illness, especially cardiovascular diseases, such as manifest arterial hypertension, severe heart disease (DCM, history of heart attack), pacemaker implantation, respiratory failure, severe active infectious disease. E-cigarette users were invited to participate in both JUUL study arms. Thus, 9 participants used both JUUL variants, 6 participants used only the "modified" JUUL, and 2 participants used only the "initial" JUUL.

Study design and questionnaires. Clinical data were collected during January and September 2020. Two appointments were scheduled for the test subjects to participate in the study. The first appointment was considered a screening, whereas the actual measurement took place at the second appointment. Usual smoking or e-cigarette consumption behavior was enquired with standardized and specially designed questionnaires.

An initial questionnaire at the screening appointment served on the one hand to assess sociodemographic data such as sex, age, weight and known pre-existing illnesses and on the other hand to assess smoking and e-cigarette consumption behavior. For example, the frequency of smoking or vaping and preferred manufacturers were assessed. At the screening appointment, physical dependence on nicotine was assessed with the Fagerström Test of Nicotine Dependence (FTND) according to Heatherton et al.[34]. No validated version of the FTND exists for e-cigarette consumption. Thus, instead of the FTND, an adapted but unvalidated questionnaire for e-cigarettes was used for participants in the JUUL study arms. The Questionnaire on Smoking Urges (QSU-G) in the German version by Müller et al. was used to assess craving[35]. QSU-G was assessed before and immediately after the vaping/smoking sessions. Further details on QSU-G and its evaluation are given in the Supplementary Information. Immediately after vaping, participants rated negative effects (side effects) of the e-cigarette on a visual analog scale (VAS) ranging from 0 (no effect) to 10 (strongest effect). The VAS used in this study was based on the one used in previous studies on e-cigarettes[36,37] and enquired urge to vomit, nausea, perspiration, headaches, palpitations, cold hands or feet, salivation, dizziness, irritation of the throat or mouth, and lightheadedness.

E-cigarettes were weighed (MC 1, Sartorius, Göttingen, Germany) before and after the measurement to determine the liquid consumption. Nicotine doses were calculated by considering liquid density (1.16 g/cm^3) and the measured nicotine concentration in the respective e-liquid (17.69 mg/mL for "modified" JUUL and 17.20 mg/mL for "initial" JUUL)[32].

Puff topography. The consumption sessions were carried out according to Fig. 1. A puff duration of 3 s was selected according to a recent study on pod e-cigarettes[38] and to a well-established regime for machine puffing of e-cigarettes[39]. It was ensured that the puffs were 3 s long and that the blood samples were taken after the completed puff. In total, 10 puffs were taken, heart rate and blood pressure were measured 4 times, and 9 blood samples were taken. A metronome was used to standardize the duration of the inhalations by providing an acoustic signal at the beginning and end of inhalation. The study investigator instructed all study participants to inhale in exactly the same way at each inhalation and study visit. Participants were instructed to inhale the product aerosols into their lungs.

Blood sampling. A peripheral venous cannula was inserted to allow blood samples to be taken at short intervals. To determine nicotine, cotinine, and *trans*-3′-hydroxycotinine (hydroxycotinine) levels, a total of nine blood samples with 7.5 mL each were taken at various time points before, during and after smoking/vaping as presented in Fig. 1. They were carried out in accordance with the generally applicable hygienic standards using Safety Multifly cannulas and S-Monovettes. Blood was placed on ice immediately after sampling until centrifugation (10 min, 1500*g*, 4 °C). Internal standard mix (10 µL of 500 ng/mL nicotine-d_3, cotinine-d_3, hydroxycotinine-d_3 in acetonitrile) was added to plasma samples (990 µL).

Analysis of nicotine, cotinine, and hydroxycotinine plasma concentrations. Nicotine and its main metabolites cotinine and hydroxycotinine were analyzed using LC–MS/MS with a validated method as published previously[40]. Blood samples were centrifuged at 1500*g* and 4 °C for 10 min to obtain plasma. 990 µL plasma was spiked with 10 µL internal standard mix (500 ng/mL (±)-nicotine-(methyl-d3), (±)-cotinine-(methyl-d3), and *trans*-3′-hydroxycotinine-d_3 in acetonitrile) at LMU in Munich and shipped on dry ice to the BfR in Berlin. For protein precipitation, 100 µL ice-cold methanol were added to 50 µL plasma sample

and then centrifuged at 4 °C and 14,000*g* for 15 min (Centrifuge 5427 R, Eppendorf, Wesseling-Berzdorf, Germany). Supernatant was diluted 1:1 with mobile phase A (see below) prior to injection of 25 µL into the LC–MS/MS system (LC: Prominence series from Shimadzu, Kyoto, Japan; MS/MS-System: API4000QTrap, AB Sciex, Framingham, MA, USA). For separation, a Luna Phenyl-Hexyl Column (150 mm length × 4.60 mm I.D., 3 µm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) with an according guard column (Phenomenex, Torrance, CA, USA) was used at 45 °C at a flow rate of 1 mL/min. As eluent A, 5 mM ammonium acetate in water, pH adjusted to 4.50 ± 0.02 with formic acid was used and for eluent B methanol. The gradient was as followed: Started at 10% B, increase for 1 min to 30% B, hold for 1 min, increase for 2 min to 95% B, hold for 2 min, decrease for 0.2 min to 10% for 0.2 min, and a hold for 2.8 min. ESI–MS/MS parameters are provided in the Supplementary Information. Nicotine, cotinine, and hydroxycotinine were quantified using a matrix matched calibration.

Machine vaping. To mimic vaping, 10 puffs were taken from JUUL devices equipped with 5 freshly opened "modified" JUUL pods or 4 freshly opened "initial" JUUL pods using a linear vaping machine for e-cigarettes (LM4E with PM1 piston pump, Borgwaldt, Hamburg, Germany). By the time the machine vaping part was performed, "initial" pods were already taken off the market. Thus, only 4 pods could be analyzed. CORESTA Reference Method 81 was applied: puff duration 3 s, puff frequency 30 s, puff volume 55 mL, rectangular puff profile[39]. Emissions were collected on glass fiber filter pads (Borgwaldt, Hamburg, Germany) that were exchanged after 2 puffs during the 30 s inter-puff interval. Total particulate matter (TPM), the weight gain in the filter, was determined by weighing (LE225-0CE, Sartorius, Göttingen, Germany). Nicotine dose was calculated by dividing by liquid density (1.16 g/cm^3) and multiplication with the nicotine concentration in e-liquid (17.20 mg/mL for the initial and 17.69 mg/mL for the modified version) as previously determined[32]. As previously shown, the nicotine content in the aerosol can be calculated on the basis of the liquid consumption leading to similar results as determined with GC-FID[32]. This is in line with other studies[41]. Further, we have previously shown that weight loss of the liquid (liquid consumption) and weight gain of the glass fiber filter (TPM) are comparable[32], also in line with the literature[41].

Pharmacokinetic (PK) parameters and statistical analysis. Statistical analysis was performed with Statistical Software Program System (SPSS) version 21.0. Data derived from QSU-G were analyzed with the t-test for paired samples. Areas under the plasma concentration–time curve (AUC) were calculated after baseline correction (subtraction of C_{t0}) applying the linear trapezoid rule. C_{max} and t_{max} were the highest nicotine concentrations per individual plasma curve and the according time points. Participants were asked to stay nicotine abstinent overnight. However, nicotine PK parameters were reported with and without baseline correction (subtraction of C_{t0}) to control potential high nicotine baseline effects, possibly due to intensive smoking on the previous evening. For statistical analysis of C_{max} and AUC, geometric mean and CV were used, and a two-sided unpaired t-test was used with lognormal values to test for statistical significance. For mean plasma curves, arithmetic means of baseline corrected concentrations at each time point were calculated. Nicotine metabolic ratio (NMR) was calculated as a surrogate for CYP 2A6 metabolic activity[42,43] by dividing hydroxycotinine plasma concentration by cotinine plasma concentration at t_0 when metabolites were detected. For NMR and other participant characteristics, median and IQR were calculated. Arithmetic mean and standard deviation were used for liquid consumption, nicotine dose, and TPM.

Results

Participants. Participant characteristics such as age, sex, FTND score, nicotine metabolic ratio, and product use characteristics are summarized in Table 1. The level of physical nicotine dependence measured by the FTND score ranged from low to very severe in all three groups. In the JUUL (modified) group the level of physical dependence was low in n = 7/15, moderate in n = 2/15, severe in n = 2/15, and very severe in n = 4/15. The JUUL (initial) group showed a low level of physical dependence in n = 4/10, a moderate level in n = 1/10, and a severe level in n = 3/10 and very severe in n = 2/10. One participant in the JUUL (initial) arm did not complete the questionnaire. In the tobacco group, the level of physical dependence was low in n = 11/14, moderate in n = 2/14 and very severe in n = 1/14. The parameters "days smoked/EC used in the past 30 days", "Pods (0.7 mL) used per day", and NMR did not differ among groups. One participant in the tobacco cigarette group seemed to have not inhaled during smoking as the plasma nicotine level did not rise higher than 0.1 ng/mL (see Supplementary Information). This participant was excluded from further analysis and calculations of the below presented results. Two participants in the modified JUUL group had to be excluded for PK data analysis because relevant blood sampling time points were missing due to clogging of the cannula and the participants could not be recruited for a repetition.

Nicotine delivery from different study products. Plasma nicotine curves from all participants are displayed in Fig. 2 as spaghetti plots without baseline correction (subtraction of C_{t0}) (Fig. 2a–c), and as arithmetic means with baseline correction and 95% confidence interval (Fig. 2d). Nicotine levels for each participant and time point in addition to individual liquid consumptions can be found in the Supplementary Information. For cigarette smokers, two different plasma curve shapes were apparent: 6 cigarette smokers had C_{max} values of above 15 ng/mL with t_{max} values of approximately 6 min (Fig. 2a, black lines), 8 cigarettes smokers had C_{max} values of below 15 ng/mL with t_{max} values of about 10 min (Fig. 2a, grey lines). For some further analysis and discussion, smokers were divided according to these two plasma curve types into "low C_{max}" smokers (C_{max} < 15 ng/mL) and "high C_{max}" smokers (C_{max} > 15 ng/mL). Median FTND score of "high C_{max}" smokers was with 1.5 (IQR 0–4.75) slightly higher than of "low C_{max}" smokers" with 0.5 (IQR 0–1). Further, NMR was with 0.59 (IQR 0.46–0.66)

Age, median (IQR)	28 (25–33)
Sex, female, n (%)	13 (41.9)
Sex, male, n (%)	18 (58.1)
Tobacco cigarette group, median (IQR), n = 14	
Fagerstrom test for nicotine dependence (FTND) before cigarette use	1 (0–2.5)
Cigarettes smoked per day when joining the study	8 (5–11)
Cigarette smokers: days smoked in the past 30 days	28 (25–30)
Nicotine metabolic ratio	0.47 (0.29–0.62)
JUUL (modified) group, median (IQR), n = 15	
Fagerstrom test for nicotine dependence (FTND) before JUUL use	3 (2–7)
Pods (0.7 mL) used per day when joining the study	0.9 (0.3–1.3)
Nicotine metabolic ratio	0.39 (0.27–0.49)
JUUL (initial) group, median (IQR), n = 11	
Fagerstrom test for nicotine dependence (FTND) before JUUL use	4 (1.5–5.75)
Pods (0.7 mL) used per day when joining the study	1 (0.5–1.3)
Nicotine metabolic ratio	0.43 (0.26–0.51)
All JUUL users, median (IQR)	
JUUL (both) users: days EC used in the past 30 days	30 (30–30)

Table 1. Participant characteristics.

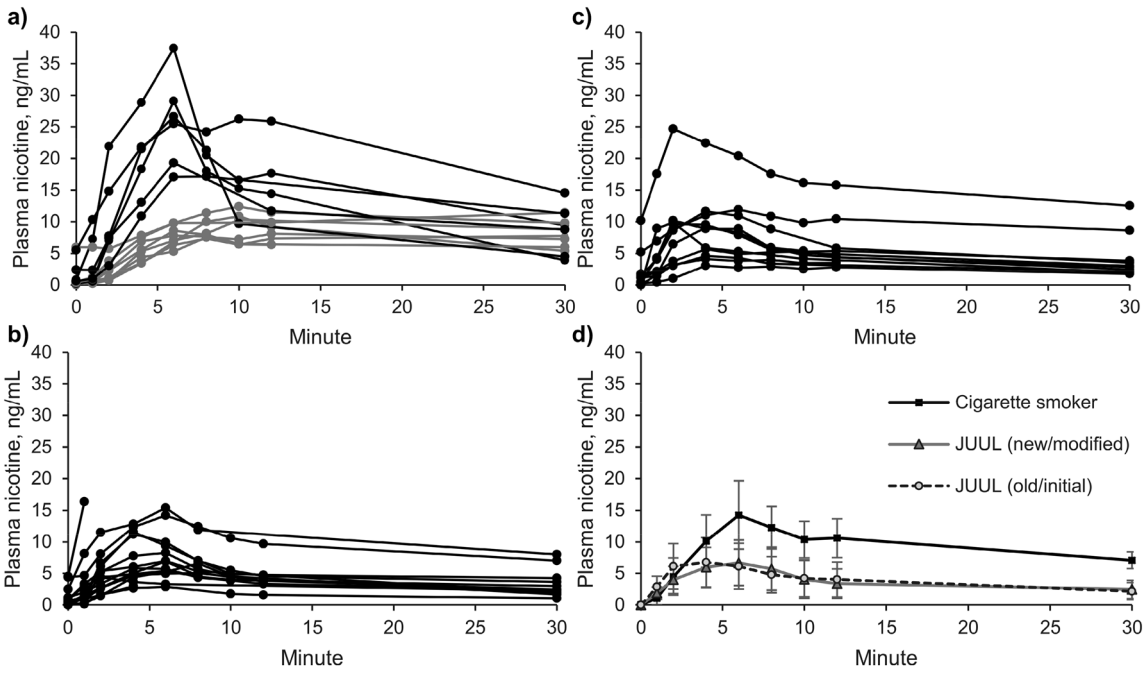

Figure 2. Individual nicotine plasma curves without baseline correction (C_{t0} subtraction) after use of (**a**) tobacco cigarettes (n = 14), (**b**) JUUL (modified) e-cigarettes (n = 15), and (**c**) JUUL (initial) e-cigarettes (n = 11). (**d**) Arithmetic means and 95% confidence interval of the plasma curves from three groups.

slightly higher for "low C_{max}" smokers" than for "high C_{max}" smokers with 0.34 (IQR 0.25–0.49) but not statistically significant (p = 0.1).

Relevant PK parameters such as C_{max} with and without baseline (C_{t0}) correction, AUC, t_{max}, liquid consumption, and calculated nicotine doses are shown in Table 2. Differences between both JUUL variants are small and non-significant for all PK parameters. Further, liquid consumption was the same for both JUUL variants. C_{max} and AUC after JUUL consumption were approximately 40–50% smaller than after tobacco smoking ($p < 0.005$). Plasma curves for metabolites cotinine and hydroxycotinine are shown in the Online Supplementary Information.

Craving. Craving factors determined with the QSU-G were divided into factor 1 for positive reinforcement and factor 2 for negative reinforcement. Two participants did not return their questionnaires. Results are shown in Fig. 3. Mean of factor 1 (positive reinforcement) decreased after tobacco cigarette smoking by 0.83, decreased

	Tobacco cigarette n = 14	JUUL (modified) n = 13	JUUL (initial) n = 11	JUUL (modified) vs. tobacco cigarette
C_{max} (ng/mL) without C_{t0} correction	14.4 (73%)	7.2 (74%)	8.1 (81%)	50% (p = 0.002)
C_{max} (ng/mL) with C_{t0} correction	13.1 (77%)	6.3 (69%)	6.5 (79%)	48% (p = 0.001)
$AUC_{0-30\ min}$ (ng/mL min) with C_{t0} correction	257.0 (49%)	103.3 (63%)	110.9 (49%)	40% (p = 0.00005)
t_{max} (min)	8 (6–30)	6 (2–8)	4 (2–6)	
Liquid consumption (mg)	N/A	31.9 ± 8.3	30.6 ± 10.9	
Nicotine dose (mg)	N/A	0.49 ± 0.13	0.47 ± 0.17	

Table 2. Relevant PK parameters for the different study products and a comparison between the "modified" JUUL version and tobacco cigarettes. C_{max} (with and without C_{t0} correction) and AUC: Geometric mean and coefficient of variance (CV%); t_{max}: Median and range; Liquid consumption and nicotine dose: Arithmetic mean and standard deviations (SD); p-values obtained with unpaired, two-sided t-test with logarithmic values.

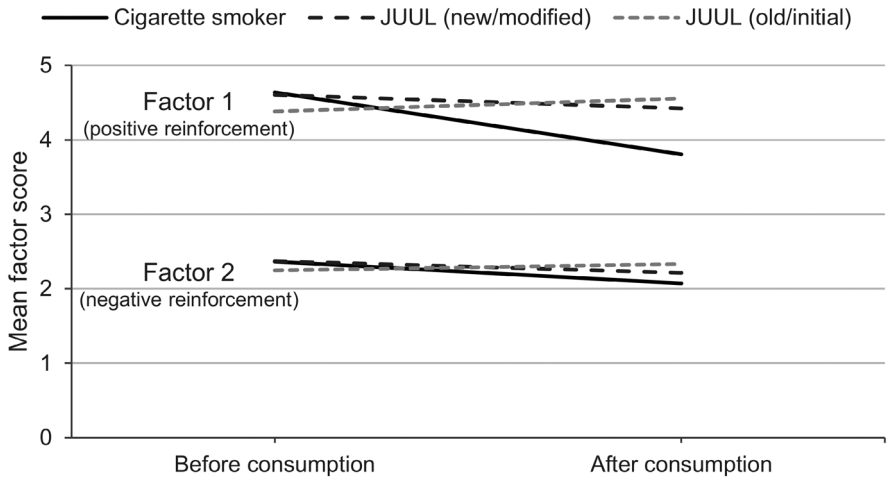

Figure 3. Mean scores of urge to smoke or urge to vape before and after consumption for all three product groups [combustible cigarette (n = 14), "modified" JUUL (n = 14), "initial" JUUL (n = 10)] divided into factor 1 (positive reinforcement) and factor 2 (negative reinforcement).

after "modified" JUUL use by 0.18, and increased by 0.17 after "initial" JUUL use. The changes in factor 1 were only significant in the tobacco cigarette group (p = 0.006).

Factor 2 (negative reinforcement) decreased in the tobacco cigarette smoke by 0.29 and in the "modified" JUUL group by 0.15. Factor 2 increased slightly in the "initial" JUUL group by 0.08. "High C_{max}" smokers showed a decrease of 1.62 in factor 1 and 0.77 in factor 2. Decrease in factor 1 in "high C_{max}" smokers was significant (p = 0.001). Factor 1 decreased in "low C_{max}" smokers by 0.23 and factor 2 increased slightly by 0.06. Survey was not fully completed by 1 participant in the "modified" JUUL arm and from 2 participants in the "initial" JUUL arm.

Side effects. Negative side effects were enquired at the end of smoking and vaping sessions. Visual analog scale (VAS) scores are displayed in Fig. 4. VAS scale ranges from 0 (no effect) to 10 (strong effect). Most side effects occurred in cigarette consumption, where the mean overall VAS score was 2.1. The tobacco cigarette group also achieved the highest values for most individual items on the VAS score.

Acute phase. For better comparison of nicotine kinetics in the acute phase, arithmetic means of baseline corrected plasma nicotine concentrations are displayed in Fig. 5 for all three study groups (smokers and users of both JUUL variants) and additionally for the two smoker subgroups ("low C_{max}" and "high C_{max}" smokers). While the means of nicotine plasma curves from cigarette smokers, especially from all smokers and from "high C_{max}" smokers, were rising for at least 6 min, mean nicotine plasma curves from JUUL users flattened after 2–4 min.

JUUL use simulation with a vaping machine. In contrast to combustible cigarettes, both versions of JUUL did not facilitate a continuous rise of nicotine plasma levels during vaping. To investigate whether this was linked to a decrease in vapor generation, a machine vaping experiment was performed. Using a standard puffing protocol (55 mL puff volume, 3 s puff duration, 30 s puff interval, rectangular puff profile), vapor generation in

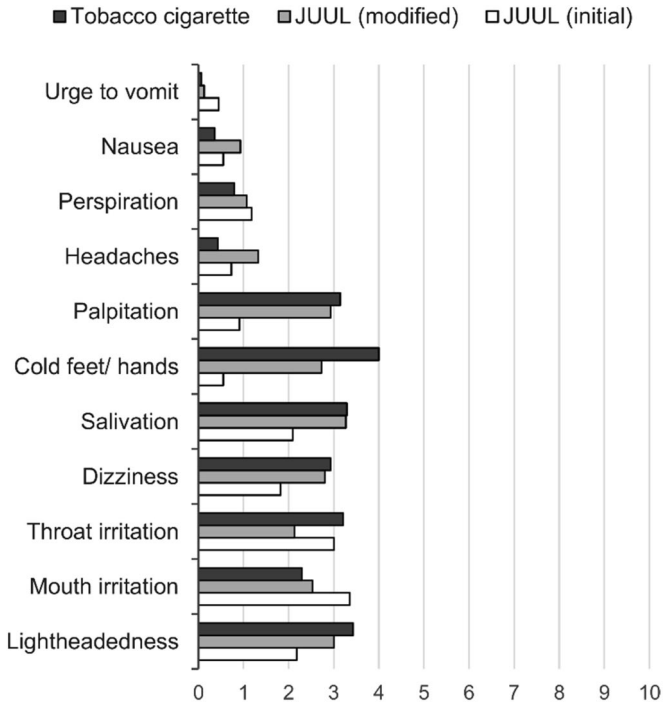

Figure 4. Reported side effects after use of tobacco cigarettes (n = 14), modified JUUL (n = 15), and initial JUUL (n = 11) version on a visual analog scale (VAS) ranging from 0 (no effect) to 10 (strong effect).

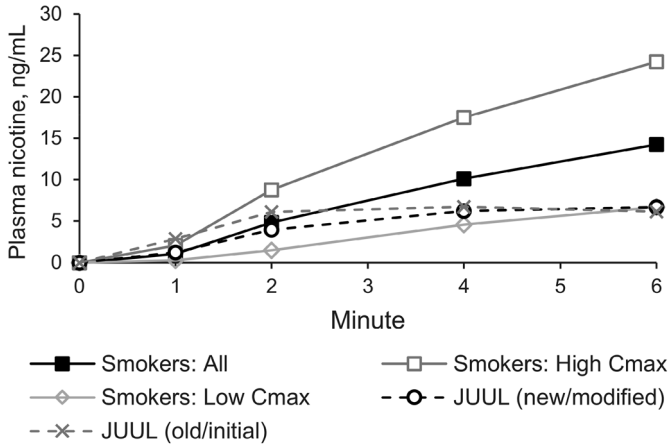

Figure 5. Plasma nicotine concentration in the acute phase. Arithmetic means of nicotine concentrations of the three study groups (tobacco cigarette smokers, JUUL (modified) users, and JUUL (initial) users) and of the smoker subgroups ("high C_{max}", $C_{max} > 15$ ng/mL; "low C_{max}", $C_{max} < 15$ ng/mL).

the first 5 min of both JUUL versions was monitored. Figure 6 shows TPM (the vapor that was collected on a filter pad) and the calculated nicotine delivery per 2 puffs for different pods. E-cigarettes were weighed before and after the procedure. The mean total liquid consumption was 30.73 ± 5.03 mg with a calculated nicotine delivery of 468.60 ± 76.65 µg for the modified version and 34.88 ± 1.04 mg consumed liquid and 517.19 ± 15.43 µg nicotine for the initial version. Divided by the puff number, average nicotine doses per puff were 47 µg and 52 µg for the modified and the initial version, respectively.

Discussion

Our study was aimed to compare performance of the two European versions of the pod e-cigarette JUUL with tobacco cigarettes under defined product using conditions. This means that puff number, interval, and duration has been standardized for all products. Only the puff volume could not be standardized. Adjustment of all parameters is necessary to limit the impact of intraindividual differences when directly comparing the performance of

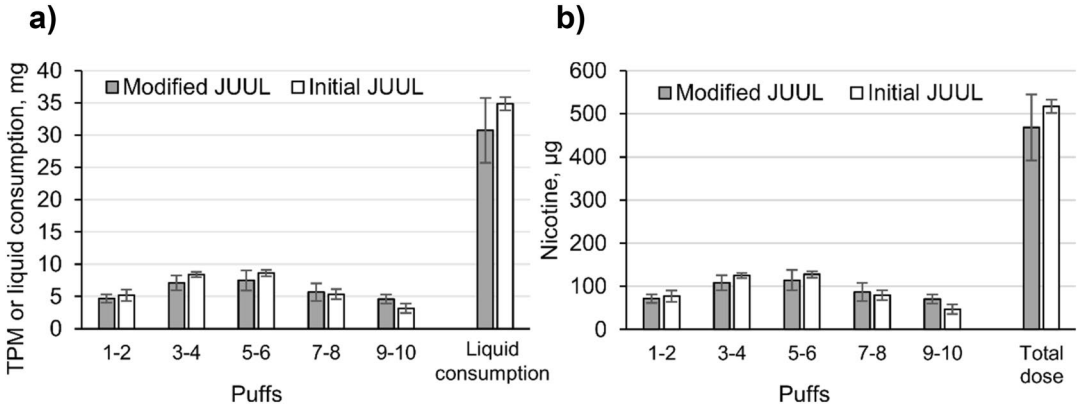

Figure 6. Machine generated vapor per two puffs expressed as (**a**) mean total particulate matter (TPM) with standard deviations for modified (n = 5) and initial (n = 4) JUUL version. Liquid consumption has been calculated by weighing pods before and after use. (**b**) Nicotine dose has been calculated by multiplying TPM with liquid nicotine concentration. Total nicotine dose was calculated using the liquid consumption.

products. Our study was designed to monitor the rise of nicotine blood concentration during the acute phase rather than to reflect real-life product use and nicotine exposure[17]. Data obtained with the pre-directed puffing protocol demonstrate that nicotine delivery from the European version of JUUL e-cigarettes is significantly lower in comparison to tobacco cigarettes: Maximum plasma nicotine concentrations and $AUC_{0-30\ min}$ from JUUL users were significantly lower (40–50%) and nicotine curves flattened earlier in the acute phase. The acute phase has been defined in a previous publication as the first 5 min during consumption[17] and is of special relevance when evaluating addictiveness of different products. Increases of nicotine concentrations in the brain are fastest upon inhalation. Consequently, nicotine inhalation leads to a rush of nicotine in the brain during the initial smoking phase[3].

These findings are in agreement with the recently published study led by Phillips-Waller et al. who compared EU JUUL (20 mg/mL), US JUUL, and tobacco cigarettes[33]. As the initial JUUL version was declared to contain 20 mg/mL while the modified version was labelled with 18 mg/mL[32], it is assumed that Phillips-Waller et al. have used the initial version in their study. They have demonstrated that the US American version with 59 mg/mL delivers comparable amounts of nicotine as tobacco cigarettes in experienced e-cigarette users[33]. The authors have reported a slightly lower nicotine delivery after EU JUUL consumption compared with our results[33]. A recent study by the manufacturer came to lower nicotine deliveries by the European JUUL versions in a study with 5-min ad libitum use sessions and pre-directed use sessions that were similar to the ones in this study[44]. Results from different studies on the same product can differ depending on the study design, especially in terms of user experiences. Inexperienced vapers using the high nicotine US JUUL version did not reach the nicotine delivery level of tobacco cigarettes[21,22].

For comparison of JUUL with other e-cigarette types, some key features like convenience of use are of relevance. For example, ease of product use was the main reason for the JUUL use continuation in college students[27]. Disposable JUUL pods are sold prefilled with e-liquid and with the heating element included. Modern pod systems with high nicotine contents have the potential to deliver comparable nicotine levels as combustible cigarettes. This marks an important distinction to earlier generation disposable e-cigarettes, so-called cigalikes that delivered nicotine less efficiently in a study using a similar puffing protocol[17]. Nicotine delivery of JUUL e-cigarettes was higher compared to cigalikes (C_{max} = 5.5 ng/mL) and was almost comparable to tank e-cigarettes (C_{max} = 9.3 ng/mL) that are more complex in handling[17].

Another main question of this study was to follow up whether the modification of JUUL pods by the manufacturer led to an increase of nicotine delivery during consumption. The initially sold European JUUL version had similar design features as the US version except for the approximately threefold lower nicotine contents. In a previous study, it was shown that the wick material in the heating element was exchanged in a modified product version that was launched in summer 2019[32]. The new wick material supposedly has a better capability to supply the heating coil with fresh liquid, resulting in a more stable and a threefold higher vapor generation compared with the initially used wick material[32]. In our study, we have raised the question whether this technical modification translates into an increase of nicotine plasma levels, consequently circumventing the nicotine limits set by the TPD. We have compared nicotine delivery during use of both European JUUL versions. Surprisingly, nicotine delivery and liquid consumption were the same for both variants. In the meantime, influence of wick material has been investigated in a clinical study by the manufacturer resulting in similar findings[44]. To follow up this observation, we simulated the pre-directed puffing protocol (10 puffs, 3 s puff duration, 30 s puff frequency) with a vaping machine, applying the CORESTA Recommended Method 81 (55 mL puff volume and rectangular puffing profile)[39]. Both EU JUUL variants were tested under the same conditions as in the clinical part. Ten puffs were drawn from each pod that was freshly opened. Liquid consumption, generated vapor, and calculated nicotine delivery did not differ between the EU JUUL variants. The overall consumed liquid in the machine vaping experiments was comparable to the mean liquid consumption of both EU variants in the clinical study. According to a mathematical model proposed by Talih et al., puff volume does not influence the mass of

generated vapor from an e-cigarette, while puff duration is an important parameter that usually equals the heating time of the coil[41]. In our study, participants were instructed to take puffs of 3 s and were guided by an acoustic signal. Consequently, puff durations in the clinical and the vaping machine part were the same. This explains the good predictability of liquid consumption in the clinical part by the vaping machine experiment. However, this raises questions about the difference in performance that has been detected in the previous study[32]. Amounts of nicotine per puff were previously determined as 61 µg in the emissions of the modified version and 23 µg in the emissions of the initial version. In the previous study, a total of 160 puffs were drawn from each pod in sets of 20 puffs[32]. Average nicotine doses per puff calculated for the herein presented vaping machine experiment were 47 µg for the modified and 52 µg for the initial version. This indicates that performance of both variants at the first ten puffs is close to the performance of the modified version over 160 puffs (61 µg nicotine per puff). In conclusion, we postulate that the wicks are initially saturated with liquid. The disadvantage of the limited liquid supply by the initial wick material only becomes apparent after a larger number of puffs are taken. Then, liquid supply becomes a limiting factor. Vapor generation with the modified wick has been more stable over time[32], indicating that this disadvantage has been compensated. This could explain that both versions have led to the same nicotine plasma curves after consumption following the pre-directed protocol. Possibly, if more puffs were taken in the clinical part of this study, nicotine delivery by the initial version might even decrease when the liquid supply becomes slower. Nicotine delivery of the modified version is expected to increase only slightly after more puffs are taken. Calculated nicotine delivery per puff when only ten puffs are used was with 47 µg per puff 77% of the nicotine delivery per puff assessed over 160 puffs (61 µg per puff), and thus already close to maximum. This improved version still cannot mimic the nicotine delivery of tobacco cigarettes in contrast to the US version that uses the initial wick material but has a threefold higher nicotine content in the liquid[32]. Interestingly, influence of wick material on puff generation is usually a neglected factor[41] as it is rather uncommon that the supply to the coil by the wick becomes rate limiting. However, the present case demonstrates that predictability of nicotine delivery can be hampered by unexpected design features that can be of advantage or disadvantage for vapor generation. This should be kept in mind for future investigations on nicotine delivery by devices with uncommon design features.

Besides the nicotine delivery, the urge to smoke and to vape was scored, and side effects were assessed. Assessment of craving was divided into positive and negative reinforcement factors[35]. Positive reinforcement describes the intention to smoke and anticipation of positive effects from smoking. Negative reinforcement indicates the craving for smoking and anticipation of relief from negative effects of nicotine withdrawal[35]. Positive reinforcement factors have been reduced for smokers but not for JUUL users. This agrees with the absence of a notable nicotine peak in the plasma curves derived from JUUL users. Decrease in factors for negative reinforcement was overall low and comparable between cigarette smokers and JUUL users. This low decrease could be linked to the overall low physical dependence of the participants according to FTND scores (see Table 1). Negative side effects were overall low and did not markedly differ among groups. Of special interest were effects such as mouth and throat irritation. Nicotine salt formulations are actually applied to alleviate the irritative effects of high nicotine contents in e-cigarette liquids[45,46]. In the present study, no notable differences were detected between groups in terms of irritative effects.

Further, it should be noted that two different plasma curve shapes were visible among cigarette smokers although the same cigarette brand was used by all participants. Smokers were divided into two subgroups solely based on visible differences in their plasma curve shapes. "High C_{max}" smokers showed a plasma curve that is known from smokers with a high rise of blood nicotine levels in the acute phase. C_{max} in all these curves was above 15 ng/mL. "Low C_{max}" smokers revealed a more plane curve and a C_{max} of below 15 ng/mL. These participants did not take advantage of the cigarette's full potential. This could have been influenced by the pre-defined puffing regimen that might be too different from their normal smoking behavior or could have been linked to the low physical dependence score that smokers, especially "low C_{max}" smokers, had in the FTND. Further, NMR was higher in "low C_{max}" smokers. Higher NMR means faster nicotine metabolism[42,43]. However, these differences were not statistically significant. Plasma curves derived from JUUL were comparable to those of "low C_{max}" smokers.

Taken together, the presented results suggest that European JUUL has a lower nicotine delivery in total and in the acute phase in comparison to tobacco cigarettes and the US version (59 mg/mL nicotine) in experienced users despite the product modification. This is in line with the recently published results[33,44]. According to these data, it becomes likely that abuse liability and addictiveness of the European version is lower. This might be one reason why high acceptance noticed in young non-smokers in the US did not become apparent in Europe.

Limitations and outlook. During 59 from 405 blood samplings, the cannula clogged that was used to draw blood. If possible, a new cannula was placed but some time points were missed. When the blood sampling at expected t_{max} was missing, the participants were contacted for a revisit. Four participants responded and were reinvited. Further, differences in plasma curves between groups of participants could have been influenced by factors such as physical nicotine dependence as measured with FTND and self-titration. Results of studies like this one are highly dependent on the recruitment of participants with different target nicotine blood levels. Although the number of participants was common for this type of study, larger numbers of participants would have helped to avoid the impact of interindividual differences in dependence. A cross-over design would have been beneficial for statistical analysis but would rely on dual users as participants should be experienced with their study product. Further, the study design enforced a vaping pattern that could differ from individual preferences of users. The aim of this study was to compare nicotine delivery by different products versions of JUUL under defined conditions. It was further discussed that JUUL pods have an inconsistent performance depending

on puff number. Thus, a long-time ad libitum consumption study could therefore give further insights to the product's potential nicotine delivery characteristics.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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Author contributions

N.M., A.R., and S.G. prepared the original manuscript draft. N.M., A.R., P.L., C.H., F.H.S., A.L., and T.R. worked on the conceptualization, N.M. and A.R. worked on the project administration. The investigation was carried out by N.M., A.R., and S.G. The study was supervised by S.K., G.K., M.K.P., O.P., A.L., and T.R. All authors have reviewed the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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2.2. Heated tobacco products

2.2.1. Levels of selected analytes in the emissions of “heat not burn” tobacco products that are relevant to assess human health risks

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

Online Supplementary Material is presented in **Annex IV**.

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LETTER TO THE EDITOR, NEWS AND VIEWS

Levels of selected analytes in the emissions of "heat not burn" tobacco products that are relevant to assess human health risks

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Abstract

Consumers of combustible cigarettes are exposed to many different toxicologically relevant substances associated with negative health effects. Newly developed "heat not burn" (HNB) devices are able to contain lower levels of Harmful and Potentially Harmful Constituents (HPHCs) in their emissions compared to tobacco cigarettes. However, to develop toxicological risk assessment strategies, further independent and standardized investigations addressing HPHC reduction need to be done. Therefore, we generated emissions of a commercially available HNB product following the Health Canada Intense smoking regimen and analyzed total particulate matter (TPM), nicotine, water, aldehydes, and other volatile organic compounds (VOCs) that are major contributors to health risk. We show that nicotine yield is comparable to typical combustible cigarettes, and observe substantially reduced levels of aldehydes (approximately 80–95%) and VOCs (approximately 97–99%). Emissions of TPM and nicotine were found to be inconsistent during the smoking procedure. Our study confirms that levels of major carcinogens are markedly reduced in the emissions of the analyzed HNB product in relation to the conventional tobacco cigarettes and that monitoring these emissions using standardized machine smoking procedures generates reliable and reproducible data which provide a useful basis to assess exposure and human health risks.

Keywords Heat not burn · Smoke chemistry · Nicotine · Non-cigarette tobacco products · Carcinogens

Abbreviations

FCTC	Framework convention on tobacco control
FDA	Food and Drug Administration
HNB	Heat not burn
HPHC	Harmful and Potentially Harmful Constituents
ISO	International Organization for Standardization
NFDPM	Nicotine-free-dried particulate matter
THS2.2	Tobacco Heating System 2.2
TPM	Total particulate matter
VOCs	Volatile organic compounds
WHO	World Health Organization

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Tobacco consumption remains one of today's major health hazards and was responsible for more than one in ten deaths in the year 2015 (GBD 2015 Tobacco Collaborators 2017). Consequently, tobacco control was strengthened by multiple measures in recent years, partly driven by implementation of the WHO Framework Convention on Tobacco Control (FCTC) (World Health Organization 2018). One strategy of tobacco companies to adapt to growing public and political pressure for further restrictions is the development of modified risk products or alternate tobacco products that are implied to be less hazardous. These claims are often based on reduced toxicant levels in the emissions, although these data cannot be directly translated into a health risk reduction. Notably, toxicant reduction strategies had also been proposed by WHO (World Health Organization 2014), opening discussions about feasibility of benefits for both smoking populations and individual smokers.

In principle, the conventional cigarettes are highly engineered products. A burning cigarette can be regarded as a connection of endo- and exothermic combustion systems (Baker et al. 2004). Yet, it gains complexity, since multiple mechanisms affect the generation of smoke (Muramatsu

2005). Smoke constituents are generated according to a temperature gradient depending on exothermic combustion within the burning tip. During puffs, temperatures can reach up to 950 °C. The majority of compounds, however, are formed in endothermic reactions within the adjacent pyrolysis-distillation zone where temperatures decrease from approximately 600 to 200 °C (Baker et al. 2004). Cigarette smoke consists of approximately 4800 compounds (Rodgman and Green 2003). At least 69 carcinogens had been identified by the year 2000 (Hoffmann et al. 2001) with an update to 98 hazardous components in 2011 (Talhout et al. 2011). Fowles and Dybing proposed an approach for prioritization of tobacco smoke constituents by applying toxicological risk assessment methods. They identified 1,3-butadiene and other substances like acetaldehyde as major contributors to cancer risk and thus suggested that harm reduction efforts should set a special focus on volatile organic compounds (Fowles and Dybing 2003).

Attempts to reduce the toxicity of tobacco smoke can be traced back to the 1960s. The initial strategies aimed for the reduction of specific compounds with ambiguous effects on overall toxicant levels (Baker et al. 2004). Further strategies to reduce toxicant levels included filter tips, filter perforation, as well as technical features such as porosity of cigarette paper and tobacco processing (Hoffmann et al. 2001). Although nicotine and tar content have decreased by more than 60% since the 1950s, this trend could not be linked to a drop in mortality rates among smokers. Furthermore, proliferation of low-yield cigarettes became a highly controversial issue. Despite the lower tar and nicotine contents, toxicant exposure has even increased when smoking intensities and profiles of long-term smokers are considered (Hoffmann et al. 2001). Further means to reduce the toxicity of tobacco smoke are limited, because combustion and consequently pyrolysis and distillation cannot be avoided in the conventional cigarettes. Since most hazardous compounds in tobacco smoke are formed between 200 and 700 °C, lower temperatures would limit formation of noxious compounds. Although earlier "heat not burn" (HNB) devices failed to gain consumer acceptance (Caputi 2016), these systems provide some advantages in terms of toxicant reduction compared to the conventional cigarettes (Henkler and Luch 2015).

First, in contrast to low-yield cigarettes, reduction of tar and associated toxicants is not necessarily interlinked with lower nicotine levels. Therefore, an increased consumption aimed at compensating deficient nicotine delivery becomes unlikely. Second, the previous reports indicate that far lower levels of relevant carcinogens can be achieved in newly developed HNB devices. One novel product referred to as "Tobacco Heating System 2.2" (THS2.2) is currently marketed in more than twenty countries. The manufacturer has stated that the yield of harmful and potentially harmful constituents (HPHC) is reduced by about 90% compared to the 3R4F reference cigarette. Importantly, a reduction of more than 95% was reported for major carcinogens, including benzene and 1,3-butadiene, when emissions were generated using the Health Canada Intense smoking regimen (Schaller et al. 2016).

From the perspective of risk assessment, it is essential to verify levels of toxicants including nicotine that can be reliably achieved in novel or modified tobacco products. It needs to be clarified whether standardized machine smoking procedures and standardized analytical methods lead to reproducible data that can be used to compare devices and to define a standard to be met if reductions were recognized as relevant. This is also an important prerequisite to address the issue of putatively modified health risks or to provide a differentiated risk assessment according to product features and specifications. However, independent investigations are scarce and urgently required. We have, therefore, analyzed the mainstream smoke emitted by THS2.2 products using different variants of commercially available tobacco sticks. This study was focused on the group of carcinogenic volatile organic compounds and aldehydes in particular according to the prioritization framework proposed by Fowles and Dybing (2003). The acquired data provide an important basis to address health risks and potential benefits in terms of a potentially reduced exposure to toxicologically relevant constituents.

Four tobacco heating devices and two different tobacco stick variants were analyzed with an LM4E smoking machine (Borgwaldt, Hamburg, Germany) following the Health Canada Intense smoking regimen (Health Canada 2000). Detailed description of analytical procedure can be found in the Supplementary Material. An overview of the measured levels of analytes in the emissions of the two different tobacco stick variants is given in Table 1. The obtained values from all used devices were pooled. We compared our emission findings to levels in mainstream smoke of different combustible cigarettes, including low and high tar, slim, and reference cigarettes, that were published by Counts et al. (2005). We displayed the lowest and the highest yields per analyte that could stem from different brands and calculated the corresponding reductions of our findings as averages of both stick variants. The levels of nicotine in this study were lower compared to the data provided by the manufacturer (Schaller et al. 2016) and also lower but still in the same range compared to the conventional cigarettes (Counts et al. 2005). Total particulate matter (TPM) was comparable to the manufacturer's findings and higher than TPM from some combustible cigarettes. The yields of the carbonyl compounds formaldehyde, acetaldehyde, acrolein, and crotonaldehyde were, with a reduction of 80–96%, considerably lower when compared to combustible cigarettes (Table 1) and comparable to the published emissions observed by the

Table 1 Levels of analytes in the mainstream smoke of two different tobacco heating stick variants with "*n*" representing the number of replicates

Parameter	Unit	Stick variant 1		Stick variant 2		Combustible cigarettes (Counts et al. 2005)	Reduction
		Mean ± SD	*n*	Mean ± SD	*n*	Min–max (mean ± SD)	%
Puff count	Puff/stick	12 ± 0		12 ± 0		5.5 ± 0.3–13.6 ± 0.5	
TPM	mg/stick	52.6 ± 3.2	24	51.2 ± 3.2	24	27.5 ± 2.4–60.9 ± 3.3	
Nicotine	mg/stick	1.1 ± 0.1	24	1.1 ± 0.1	24	1.07 ± 0.06–2.70 ± 0.14	
Water	mg/stick	31.7 ± 5.5	24	28.5 ± 4.6	24	9.82 ± 1.42–21.35 ± 2.23	
NFDPM	mg/stick	19.8 ± 6.5	24	21.6 ± 5.9	24	16.3 ± 1.3–37.6 ± 2.1	
Acetaldehyde	µg/stick	179.4 ± 10.5	18	183.5 ± 10.1	14	930 ± 85–1540 ± 153	80.5–88.2
Acrolein	µg/stick	9.9 ± 1.2	18	8.9 ± 1.0	14	89.2 ± 7.3–154.1 ± 13.6	89.5–93.9
Formaldehyde	µg/stick	5.3 ± 0.4	18	4.7 ± 0.3	14	29.3 ± 3.8–130.3 ± 10.8	82.9–96.2
Crotonaldehyde	µg/stick	< 3.0	18	< 3.0	14	32.7 ± 1.5–70.8 ± 9.0	
1,3-Butadiene	µg/stick	0.22 ± 0.02	6	0.20 ± 0.02	6	77.0 ± 4.8–116.7 ± 14.3	99.7–99.8
Benzene	µg/stick	0.63 ± 0.07	6	0.54 ± 0.05	6	49.7 ± 7.7–98.3 ± 4.3	98.8–99.4
Isoprene	µg/stick	2.10 ± 0.35	6	1.82 ± 0.24	6	509 ± 41–1160 ± 65	99.6–99.8
Styrene	µg/stick	0.47 ± 0.06	6	0.49 ± 0.09	6	15.4 ± 0.8–33.3 ± 2.8	96.9–98.6
Toluene	µg/stick	2.15 ± 0.37	6	1.96 ± 0.23	6	86.2 ± 11.0–176.2 ± 15.7	97.6–98.8

Yields are compared to lowest and highest levels found by Counts et al. in combustible cigarettes

All levels were generated using HCI smoking regime

TPM total particulate matter, *NFDPM* nicotine-free-dried particulate matter

manufacturer (Schaller et al. 2016). Similar to the carbonyl compounds, the emissions of the volatile and semi-volatile compounds benzene, 1,3-butadiene, isoprene, styrene, and toluene were with a reduction of 97 to over 99% markedly lower when compared to combustible cigarettes (Table 1). The range of values found is again similar to the manufacturer's data (Schaller et al. 2016). To address consistency of nicotine and TPM release during the smoking procedure, the 12 puffs of the smoking protocol were divided into four intervals of three puffs each and analyzed separately. The nicotine and TPM release was shown to be inconsistent with lower yields in the beginning. More detailed information can be found in the Supplementary Material.

For a profound assessment of health risks and putative benefits, independent studies by different laboratories are needed. Furthermore, our intention was not only to reassess emissions of HPHC and compare to other studies, but also to use standardized methods as used by surveillance authorities and establish them for this particular application. More HNB products from different manufacturers are expected to appear on a wider market in the future with claims of reduced toxicant levels. Therefore, surveillance authorities will require standardized methods for routine analysis of HNB products to verify claims and to protect consumers from being misled.

In this study, we applied methods that are based on international standards to investigate emissions of a novel HNB product. We have used a commercially available linear smoking machine that was initially developed for electronic cigarettes. Thus, the procedure can be easily transferred. Our data are in good agreement with some recent investigations. In their recent study, Li et al. analyzed a set of HPHCs, including aldehydes and VOCs, in the emissions of the same HNB product using ISO and HCI smoking regimen (Li et al. 2018). The data presented in our study support their findings and conclusions. Farsalinos et al. analyzed the nicotine delivery in the preceding HNB model of the same manufacturer (Farsalinos et al. 2017). They found a higher nicotine yield as compared with the currently marketed THS2.2 that was analyzed here. Another study that used a custom instrument and custom smoking regimen reported similar findings for aldehydes but not for nicotine (Auer et al. 2017). A recent study by Bekki et al., that used the preceding HNB model as well, focused on tobacco-specific nitrosamines (Bekki et al. 2017). Their determined levels for nicotine, TPM, and water are comparable to ours. Another group developed a headspace solid-phase microextraction-based method for semi-quantitative assessment of VOCs emitted by HNB products (Savareear et al. 2017). The issue of toxicant reduction is complex, since these calculations depend on the reference product. Importantly, our data confirm absolute values for selected toxicants in the emissions of the analyzed HNB that are in agreement with data published by the manufacturer (Schaller et al. 2016). Furthermore, our study is in agreement with the currently published FDA Tobacco Products Scientific Advisory Committee (TPSAC) briefing document (Food and Drug Administration 2018).

Another interesting point to show was that emissions of particulate matter and nicotine were not consistent during the smoking procedure. Unlike electronic cigarettes, in the European Union conventional cigarettes are not regulated to

provide consistent nicotine delivery. Although HNB products are likewise not regulated in terms of consistency of nicotine delivery, the observed inconsistent delivery may influence consumer satisfaction, nicotine blood levels, and adaptations of smoking behavior, and needs to be investigated further.

In our study, we found comparatively high levels of tar. For the conventional cigarettes, "tar" is defined as particulate matter subtracted by nicotine and water (ISO 4387:2000), and is limited to 10 mg tar per cigarette as determined with the ISO smoking regimen (ISO 3308:2012) according to European regulations (EU 2014). Importantly, the water content in the smoke of the HNB product is high compared to the conventional cigarettes, thus affecting the NFDPM calculation more than in the conventional cigarettes. The manufacturer applied a special instrumental set-up to avoid the loss of water (Ghosh and Jeannet 2014). This special equipment is neither standardized nor applicable for surveillance authorities. Therefore, we decided to use the extraction and titration method which is already applied in routine analysis.

Although the NFDPM value for HNB products can be formally calculated as for the conventional cigarettes, direct comparisons would be misleading. TPM of the conventional cigarettes, which is defined as the portion that is trapped on the filter (ISO 4387:2000), contains typical toxicants that were confirmed to be strongly reduced in the analyzed HNB product. In contrast, the proportion of humectants in NFDPM of HNB products is markedly higher compared to the conventional tobacco cigarettes.

The strongly reduced HPHC levels in the emissions of the analyzed HNB device are likely to reduce toxicant exposure. Nevertheless, it should be noted that machine smoking protocols are standardized methods aimed to monitor reliable emissions, but not accurate models for human exposure or smoking behavior. Further studies are required to address the magnitude of exposure reduction. However, the herein confirmed reductions of relevant toxicants by about 80–99% are substantial, leading to the relevant question of putatively reduced health risks. Risk assessment models need to be established that could take advantage of the framework for prioritization of carcinogens in cigarette smoke as proposed by Fowles and Dybing (2003). Mainstream smoke constituents were prioritized according to their concentrations and their cancer potency factors. A recent study performed calculations with one data set of THS2.2 and provisionally concluded cancer potencies of HNB products to be more than 10% lower than the conventional cigarettes (Stephens 2018). We could confirm a highly substantial reduction of prioritized major carcinogens, such as 1,3-butadiene, acetaldehyde, and benzene. Several studies addressed lowered health risks due to reduced smoking of tobacco cigarettes and substantial data are available (Inoue-Choi et al. 2018; Law et al. 1997; Pesch et al. 2012). It is still uncertain whether these data are applicable to model reduced exposure in relation to HNB products. Although modified health risks are expected, it is difficult to provide an estimate for both populations and individual smokers.

HNB products are a novelty to the market and more manufacturers are expected to launch new versions in this product category. Therefore, it is essential to define criteria that should be met by new products. Analytical assessment of HPHC contents in mainstream smoke can help to define these standards. Regarding a risk–benefit analysis that is required for novel tobacco products in Europe (2014/40/EU) (EU 2014), substantial reductions of toxicant levels might be regarded as a discrete benefit compared to combustible cigarette consumption, even if potential consequences for human health still need to be explored. This is consistent with the previous approaches proposed for the conventional cigarettes by WHO (World Health Organization 2014).

We propose that new HNB products need to show comparable or lower HPHC levels in the emissions as the analyzed device to confirm a benefit in the context of an overall risk assessment. The applicable values for toxicant levels should be continuously minimized and reassessed when refined products and technologies become available. By contrast, it should be considered insufficient to show only a minor decrease of HPHC levels in comparison to the conventional cigarettes. Furthermore, it should be assessed whether other levels of toxicologically relevant substances are elevated in return as already discussed for propylene glycol, glycerol, glycidol, and acetol (Food and Drug Administration 2018). Therefore, further studies need to be conducted: first, more independent assessments of toxicant yields need to be published by using standardized methods for the above discussed reasons. Second, it should be examined whether HNB products lead to other toxicants and health hazards that have been neglected so far. Finally, the long-term impact on public health needs to be assessed in the future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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2.2.2. Levels of selected analytes in the emissions of a heated tobacco product with external heating of the tobacco

Unpublished results.

The assessment of another HTP was performed in cooperation with Jürgen Hahn and his coworkers at CVUA Sigmaringen (Sigmaringen, Germany).

The aim was to quantify relevant carcinogens in the emissions of a second device that has a different mechanism for tobacco heating. The product was expected to enter the German market and knowledge gaps were addressed for a toxicological assessment. Further, the methods that were implemented to study emissions of HTPs were used to analyze a second device to test their applicability.

The studied device

Different mechanisms of heating have been developed to heat the tobacco in HTPs, many of these devices use electrical energy. This has been discussed in **1.3**. While the device analyzed in the first study on HTP emissions (**2.2.1.**) supplies heat via a heating blade that is inserted into the tobacco stick, the current device in the second study heats the tobacco stick from outside (see Figure 1 in the review article **1.3.1. Heated Tobacco Products: A Review of Current Knowledge and Initial Assessments**). According to the manufacturer, the reconstituted tobacco in the consumable is heated by a 2-zone heater in the device [162]. The 2-zone heater supposedly uses a temperature program with a maximum temperature of 245 °C [162].

Material and methods

Material and methods were used as in **2.2.1.** and described under **Annex IV: Supplementary Material**. The devices and consumables (bright tobacco flavor) were bought in Spring 2018 from tobacco retailers in Basel, Switzerland. Method for determination of VOCs was adjusted. The amount of internal standard, benzene-d_6, was reduced to 6 µg. Further, acrylonitrile (≥ 99 %, Sigma-Aldrich, St. Louis, MO, USA) was added as an analyte instead of toluene. Heating interval prior to the first puff was 40 s instead of 20 s. The device turns off after 3.5 min, thus 8 puffs can be drawn using HCI puffing regimen.

Results and discussion

Determined amounts of relevant constituents in emissions per consumable of the studied HTP are summarized in **Table 1**. Additionally, published data for tobacco cigarette emissions derived with the

HCI regime are given [175]. Further, reduction of compounds in HTP in comparison with data for combustible cigarettes were calculated.

Table 1. Levels of nicotine and selected toxicants in the mainstream emissions of a heated tobacco product with an external heating of tobacco (n = replicates, BDL = below detection limit).

		Analyzed heated tobacco product		Combustible cigarettes from Counts *et al.* [175]	Calculated reduction
Parameter	Unit	Mean ± SD	*n*	Min–max (mean ± SD)	%
Puff count	Puff/stick	8 ± 0		5.5 ± 0.3 – 13.6 ± 0.5	
Total particulate matter (TPM)	mg/stick	25.4 ± 1.4	9	27.5 ± 2.4 – 60.9 ± 3.3	
Nicotine	mg/stick	0.32 ± 0.03	9	1.07 ± 0.06 – 2.70 ± 0.14	70.1 – 88.1
Water	mg/stick	14.7 ± 1.2	9	9.82 ± 1.42 –21.35 ± 2.23	
Carbonyl compounds					
Acetaldehyde	µg/stick	90.7 ± 2.6	5	930 ± 85 – 1540 ± 153	90.2 – 94.1
Acrolein	µg/stick	3.3 ± 0.1	5	89.2 ± 7.3 – 154.1 ± 13.6	96.3 – 97.9
Formaldehyde	µg/stick	2.7 ± 0.2	5	29.3 ± 3.8 – 130.3 ± 10.8	90.8 – 95.8
Crotonaldehyde	µg/stick	< 3.0	5	32.7 ± 1.5 – 70.8 ± 9.0	> 99
Volatile organic compounds (VOCs)					
Acrylonitrile	µg/stick	0.088 ± 0.041	6	12.1 ± 1.3 – 34.3 ± 2.7	> 99
1,3-Butadiene	µg/stick	BDL	6	77.0 ± 4.8 – 116.7 ± 14.3	> 99
Benzene	µg/stick	0.054 ± 0.004	6	49.7 ± 7.7 – 98.3 ± 4.3	> 99
Isoprene	µg/stick	BDL	6	509 ± 41 – 1160 ± 65	> 99
Styrene	µg/stick	0.023 ± 0.001	6	15.4 ± 0.8 – 33.3 ± 2.8	> 99

Selected carbonyl compounds in mainstream emissions were reduced by more than 90 %, VOCs by more than 99 %. The reduction was comparable and slightly higher compared to the first studied HTP (**2.2.1.**). However, the emitted nicotine amount is much lower compared to tobacco cigarettes.

The manufacturer of the device has published results of their chemical assessment of mainstream emissions of the product [176]. The results presented herein and standard deviations are comparable to the manufacturer's assessment. While the water content in mainstream emissions was probably underestimated in the analysis of the first device, the water content of the second device was close to the manufacturer's results (12.1 ± 1.1 mg water/consumable unit) [176].

2.2.3. Online-coupled pyrolysis gas chromatography as a useful tool to identify unknown thermal degradation products from materials in heated tobacco products

Unpublished results.

Abstract

Heated tobacco products (HTP) are designed to produce an inhalable aerosol by heating tobacco at lower temperatures than conventional cigarettes. At temperatures of up to 350 °C, formation of known tobacco toxicants was shown to be reduced. However, novel tobacco products using different tobacco additives, new materials, and design features might generate alternative profiles of toxicologically relevant emissions. Uncomplicated methods that are feasible for independent laboratories are needed to screen for substances that can be formed during thermal degradation of used new materials. In the present pilot study, an HTP filter material was heated under defined conditions and thermal degradation products were analyzed using an online-coupled system of pyrolysis and gas chromatography with mass spectrometric detection. A temperature range of 200 °C to 350 °C was applied under inert and oxidative conditions. The polymer was characterized as copolymer of polylactic acid and polycaprolactone. Degradation products like acetaldehyde were identified and compared to reference standards. Pyrolysis - gas chromatography - mass spectrometry is a useful tool to study the generation of substances from different materials. Formation of unknown toxicants from materials in new HTP consumables needs to be characterized to include them in future emission studies. The online coupling approach is especially beneficial if volatile substances are generated.

Introduction

HTPs are designed and advertised to have a reduced toxicant emission in relation to cigarette smoke. To confirm this, levels of constituents with toxicological relevance in mainstream emissions need to be analyzed. The first step is the determination of putative reductions of well-known cigarette smoke constituents. Carcinogens like 1,3-butadiene, acrylonitrile, and acetaldehyde are the main contributors to carcinogenicity of smoking and have been confirmed to be reduced by approximately 80% to over 99% in the emissions of the investigated HTP (2.2.1.). Cigarette smoke constituents have been studied for decades and assessed regarding their contribution to health hazards of smoking. Priority lists of relevant analytes have been developed by WHO, FDA, and other researchers [15-18]. However, a

reduction of these constituents could be counteracted when other substances of concern are increased in turn. Most laboratories use quantitation methods that are targeted at selected analytes and are therefore blind for other substances. Consequently, substances that have a new relevance are not covered and are neglected when assessing the risks of the products. Untargeted quantitation approaches require sophisticated techniques relying on high resolution mass spectrometry [177] and are not feasible for many independent laboratories such as governmental laboratories. Therefore, other methods are needed to screen for potential new substances of interest.

At first, potential sources of relevant substances should be identified, like new materials that are used for the HTP consumables. Generation of the substances due to heating of these new materials should be characterized. A useful tool to study materials and tobacco additives under heat is online-coupled pyrolysis gas chromatography with mass spectrometric detection (Pyr-GC/MS). This method has been used to study composition of polymer materials, tattoo pigments, and was used to simulate pyrolysis of tobacco additives [178-182]. Pyr-GC/MS can reveal the composition of the new polymer material and can help to find new analytes that require a closer look. Derived degradation products from tobacco additives, or in this case used materials, should be followed up with quantitation in mainstream emissions.

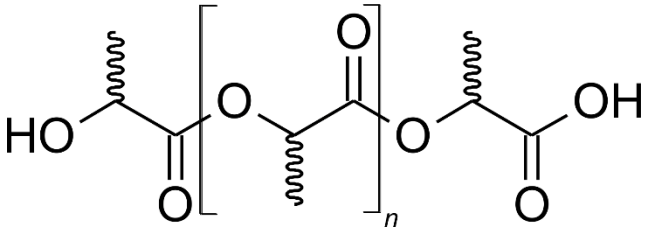

Figure 5. Structure of polylactic acid (PLA).

For example, one HTP consumable contains a filter that consists of a folded polymer sheet, presumably made of polylactic acid (PLA, structure shown in **Figure 5**). Consumers have reported that the PLA filter and the tobacco plug changed visibly after consumption, as displayed in **Figure 6**. While the tobacco plug seems to have undergone some charring processes during heating of up to 350 °C, the PLA filter shows a yellowish discoloration and material hardening after consumption. This observation is in line with findings by other researchers [183]. As a pilot study, Pyr-GC/MS is used to simulate the fate of the new filter material during thermal degradation at defined temperatures ranging from 200°C to 350°C under inert and oxidative conditions. Toxicological relevant compounds that are generated in the process are potential candidates to be included for emission tests.

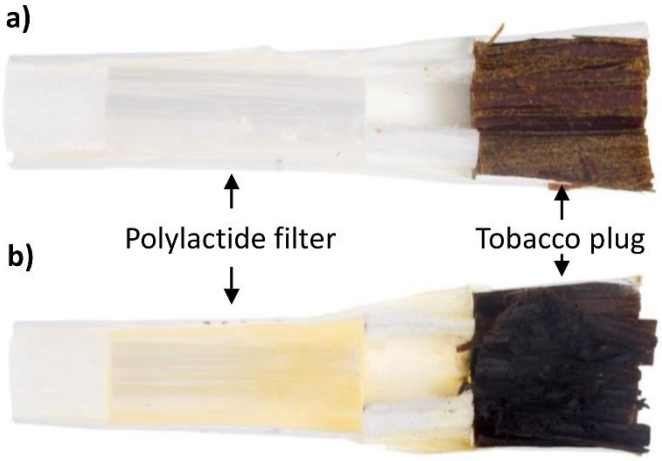

Figure 6. Halved heated tobacco consumable a) before and b) after use.

Material and Methods

Chemicals, materials, and samples

Used solvents were of analytical or higher purity grade. Poly-D,L-lactide (average Mn 20,000), L-(+)-lactic acid (80.3%), ε-caprolactone (100.0%), lactide (99.7%), triacetin (99.98%), diacetin, and formaldehyde cyanohydrin (70% in water) were purchased from Merck KGaA (Darmstadt, Germany). Acetaldehyde (99.8%) was bought from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Prior to use as a reference, chemicals were dissolved in appropriate solvents: Ethanol, methanol, dichloromethane (all from Merck KGaA, Darmstadt, Germany) or ultra-pure water (prepared with a Milli-Q Integral Water Purification System from Merck KGaA, Darmstadt, Germany). The analyzed HTP consumables were purchased in local stores in Berlin, Germany.

Online-coupled pyrolysis gas chromatography with mass spectrometric detection (Pyr-GC/MS)

Analysis was carried out on a GC-MS (7890A and 5975C, Agilent, Santa Clara, CA, USA) equipped with a multipurpose autosampler (MPS2-XL, Gerstel, Mühlheim, Germany). The polymer filters were extracted from the consumables, unrolled, and cut. Pieces of approximately 1 mm² were placed into pyrolysis tubes that were closed with quartz wool (Gerstel, Mühlheim, Germany). Tubes were placed by the autosampler into the pyrolysis module with a platinum filament that is located inside a thermal desorption unit (TDU) on top of a cooled injection system 4 (CIS) equipped with a liner filled with deactivated glass wool (all Gerstel, Mühlheim, Germany). The thermal treatment of the sample for 0.33 min at different temperatures was performed with the following instrument settings: Pyrolysis was programmed with a lead time of 0.10 min, a follow-up time of 1.00 min, an initial time of 0.33 min, and the pyrolysis temperature depending on the experiment: 200 °C, 225 °C, 250 °C, 275 °C, 300 °C, 325 °C, or 350 °C. The TDU had an initial temperature of 50 °C with a delay time of 0.50 min and an initial time of 2.00 min, followed by a 720 °C/min ramp up to 100 °C and a final hold for 1.4 min. TDU transfer

temperature was set as 120 °C. Solvent vent mode was used with a purge flow of 50 mL/min (helium, purity 99.999%, Linde, Pullach, Germany). For simulating oxidative conditions, synthetic air (20% oxygen in nitrogen; purity 99.999%; Linde, Pullach, Germany) was supplied with an apneumatic gas regulator from Gerstel (Mühlheim, Germany) as described previously [182]. During pyrolysis, analytes were cryotrapped at -120 °C in the CIS while a backflush of -1.39 mL/min (helium, purity 99.999%, Linde, Pullach, Germany) prevented oxygen from entering the GC-MS system. Oxygen was flushed back from the analytical column while the analytes were cryotrapped and held inside the CIS. Cryotrapping and backflush was enabled for inert and oxidative conditions for better comparability. After 3 min, the backflush switched off and the CIS heated to 220 °C at a rate of 12 °C/s. Subsequently, separation was performed on an HP-5MS GC column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent, Waldbronn, Germany) equipped with a guard column (10 m × 0.25 mm; Agilent, Waldbronn, Germany) and a carrier gas flow of 1 mL/min (helium, 99.999%, Linde, Pullach, Germany). The following GC oven program was used: 50 °C for 6 min, followed by a 10 °C/min ramp to 320 °C with a 3 min hold. Total ion chromatograms were collected in scan mode within the mass range of 30–500 amu. Compounds were identified using ChemStation E02.02.1431 (Agilent, Waldbronn, Germany) in combination with the NIST Mass Spectral Library 2.0 g version 11 (National Institute of Standards and Technology, Gaithersburg, MD, USA) database. Additionally, retention times and mass spectra were compared to those obtained from standard substances. A weekly performance check of the instrumentation was performed with polystyrene under inert conditions at 750 °C, as described previously [182].

Results

The PLA filter material was treated at temperatures from 200 °C to 350 °C under inert and oxidative conditions. Thermal degradation products were analyzed online with gas chromatography with mass spectrometric detection. For semi-quantitative evaluation of substance generation, peak areas of the six most relevant generated substances in the total ion chromatograms were related to the total of identified peaks. Mean values and standard deviation for four individual filters are displayed in **Figure 7**.

At temperatures between 200 °C and 275 °C, mainly the monomers of PLA (lactide) and polycaprolactone (ε-caprolactone) and a humectant (ester of glycerol) were found under both inert and oxidative conditions. This confirms that the filter is made of a copolymer consisting of PLA and polycaprolactone. A clear differentiation of the glycerol ester could not be made with this instrument, since diacetin and triacetin have similar chemical structures and analysis of reference substances resulted to peaks at the same retention times and identical spectra. Further, a conversion of both substances into the same product under the applied conditions is possible. Due to the low toxicological relevance of diacetin and triacetin, an additional differentiation with another instrument was not performed.

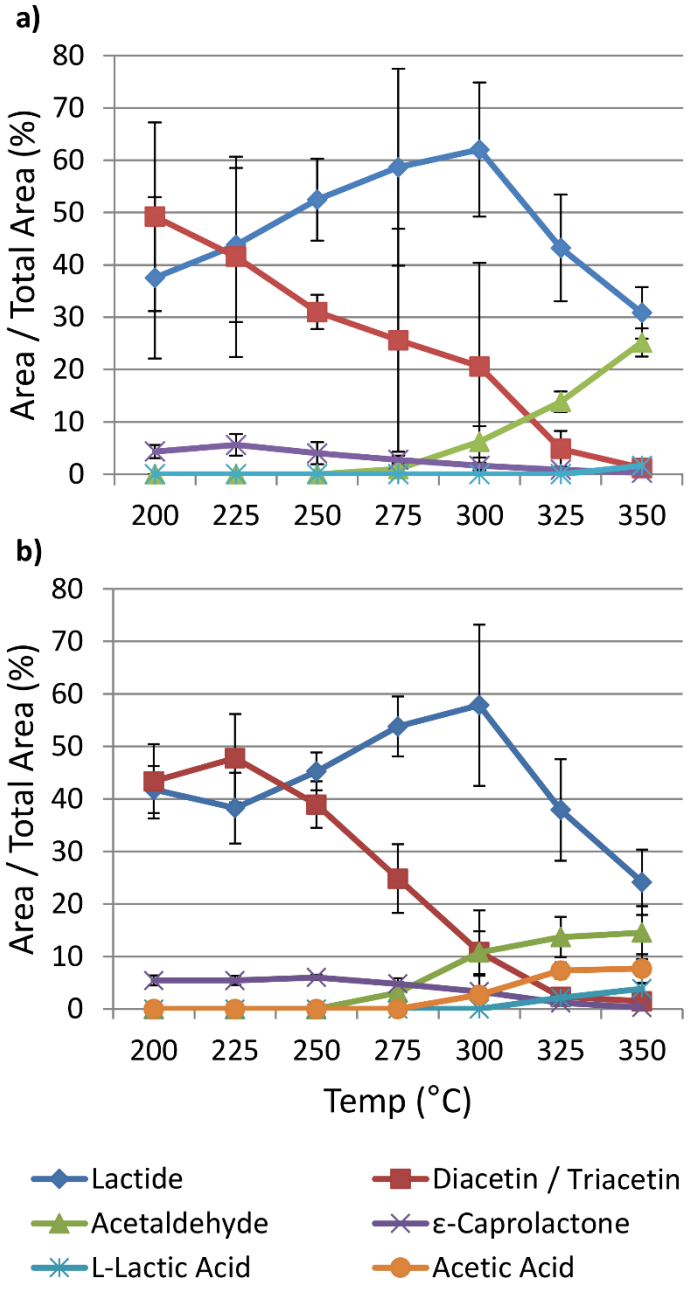

Figure 7. Proportion of peak areas of identified compounds in relation to the total peak area after thermal degradation of four individual filters under **a)** inert and **b)** oxidative conditions at different temperatures.

Between 275 °C and 350 °C, the polymer degrades also to lactic acid and acetaldehyde under inert conditions and additionally to acetic acid under oxidative conditions. Acetaldehyde is present up to 25% under inert and up to 14% under oxidative conditions at 350 °C, expressed as proportion of acetaldehyde peak area to the total of all peak areas in the chromatogram. It should be noted that this is only a semi-quantitative estimation. For example, correlation of response and concentration may be different between analytes and substances that are not GC-amendable are not detected with this method.

An example chromatogram is shown in Figure 8. The filter material was heated to 300 °C under inert conditions. The degradation products were analyzed by online-coupled gas chromatography with mass spectrometric detection. Reference substances of the compounds were analyzed, and retention times and spectra were matched. The identified compounds are labelled in the figure, and their chemical structures are presented.

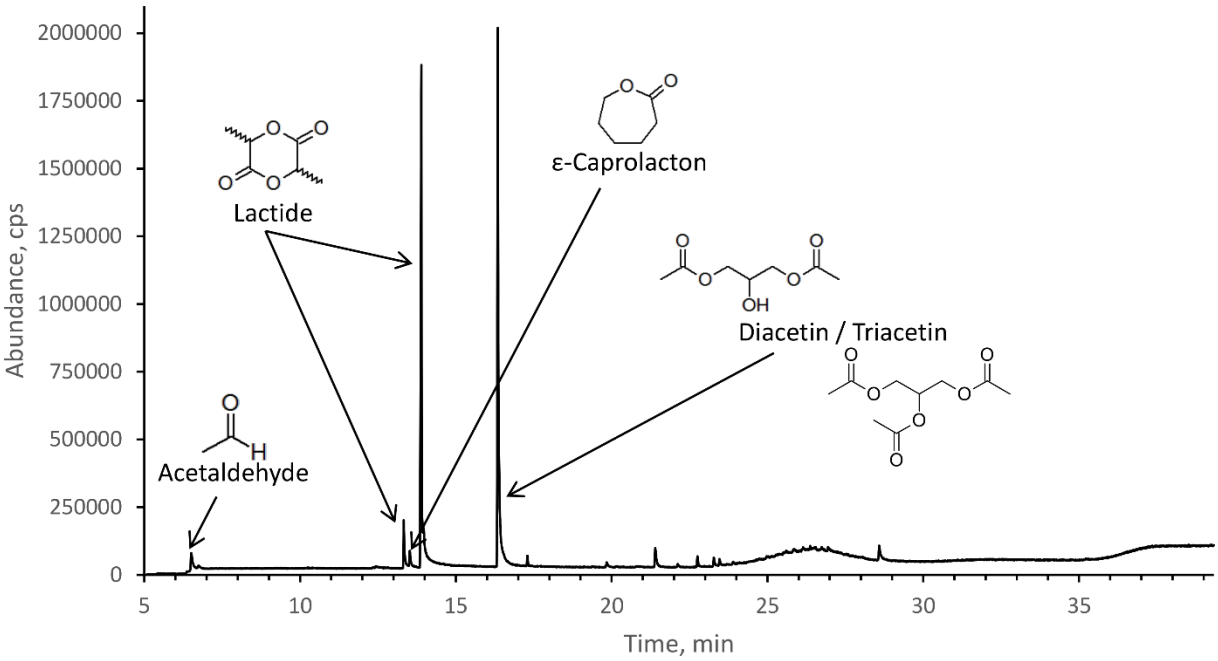

Figure 8. Chromatogram after thermal degradation of the polylactic acid filter material under inert conditions at 300 °C. Identified compounds are labelled and their chemical structures are shown.

Discussion

In the present study, the fate of the filter material was simulated at certain temperatures and conditions. Temperature-dependent formation of substances has been observed. These substances could also be generated during actual consumption and could be transferred into mainstream emissions. Thus, thermodegradation products with toxicological relevance should be added to the analyte list for chemical characterization of product emissions. Other methods have been used to determine substances that are formed from the PLA filter during consumption. Davis *et al.* have used the HTP consumable with a custom smoking machine [183]. Afterwards, they extracted the filter and analyzed pieces of it with headspace gas chromatography. They have reported the formation of formaldehyde cyanohydrin, but not acetaldehyde [183]. Formaldehyde cyanohydrin has not been detected in the present study with the Pyr-GC/MS and was not found to be a thermodegradation product of the filter. These different outcomes could be due to differences in the methodology. In the approach by Davis *et al.*, the filter material comes into contact with emissions generated from the tobacco. Such interactions, e.g., chemical reactions or adsorption, between compounds derived from

tobacco with the polymer are not assessed in the Pyr-GC/MS set-up. However, the aim of the presented study was to characterize the degradation of the filter material by itself. Another important difference is that offline techniques might underestimate volatile substances such as aldehydes. Volatiles that are generated from the filter material in the pyrolysis module are directly transferred to the online-coupled gas chromatograph after their formation and can be detected.

In this pilot study, one substance of toxicological concern has been clearly identified to be generated from the filter material. Acetaldehyde is classified as carcinogenic CARC 1B by CLP regulation [132, 133]. The substance was present at temperatures above 275 °C under inert and oxidative pyrolysis conditions. This substance would be a candidate that needed to be added to the analyte list for chemical characterization of emissions if it was not already included. In fact, acetaldehyde has been found in the mainstream emissions of this product. It showed the lowest reduction (80-90%) among all tested carbonyl compounds compared to cigarette smoke (**2.2.1.**) [174]. It is not clear at what levels acetaldehyde is generated from the filter and to what extent acetaldehyde that is generated from the filter contributes to the emissions by the complete consumable. However, other substances might be formed from new materials used in other HTP consumables. This is especially of importance if less characterized or knock-off products enter the market. Targeted chemical emission analysis will be blind for newly formed substances and untargeted approaches require expensive equipment. Thus, pyrolysis online-coupled to gas chromatography with mass spectrometric detection is a useful tool to characterize the composition of polymer materials and to simulate their behavior under defined conditions to screen for new hazards. Whether the tested temperatures are reached inside the material during actual consumption plays a minor role, as the identified substances will be later quantified under more realistic conditions in the mainstream emissions. Further, worst-case scenarios that avoid underestimating new risks are beneficial for consumer protection.

In summary, substances of toxicological concern that can be generated from HTP materials but are not yet monitored in the emissions should be added to the list of analytes to be quantified. This will help to assess the exposure of HTP consumers to hazardous substances that might have been underestimated without a prior screening of thermal degradation from new HTP materials.

3. Discussion

Science-based assessment of risks of ANDS for individual consumers and for the population requires reliable and meaningful data. The most useful data are results from epidemiological studies or long-time observations of public health effects. Decrease of smoking-associated cancer and other smoking-attributable diseases confirmed by epidemiological data is the main goal for harm reduction strategies and crucial for their success. However, these data will only be available after decades of product use. This time span is too long for regulators to decide whether to allow such products on the market or not. Clinical studies assessing biomarkers after defined periods of product use can be performed in a more realistic time frame. Such biomarkers could be surrogates for actual exposure to harmful constituents or for potential harm (e.g., changes in blood pressure or in respiratory parameters) [184-186]. Further, studies on nicotine kinetics, especially in the acute phase, are used to draw conclusions on potential addictiveness of the products [78]. The generated data are important for assessing the risk to the individual consumer and are useful to evaluate novel product groups or when important product features change. However, products of one category can have great differences in design, composition, emissions, and consequently in potential risks. Cost- and time-intensive clinical biomarker studies are not feasible to assess risks involved with all individual products on the market or for monitoring of product changes. Thus, analytical methods are needed to address remaining data gaps. Further, it is possible to perform quantitative risk assessments based on analytical data [20, 168, 169]. To evaluate the usefulness of the alternative products for harm reduction, risks are evaluated in comparison with tobacco cigarettes. Requirements for suitable methods to be used for risk assessment or surveillance of ANDS could be defined as followed:

- Methods that will be applied for surveillance of the market by official authorities should be cost- and time-effective. These methods should be realizable with equipment and instruments that is usually available in official control laboratories.
- Quantitation methods should be validated and fit for purpose for reliable analysis.
- Results of analytical methods should be correlated with outcomes of clinical studies.
- The same or analogous methods should be applicable to tobacco cigarettes to allow a comparison of emission levels.

3.1. Analytical methods for analysis of e-cigarettes and heated tobacco products

One aim of this thesis was the development of new or modification of existing analytical methods for risk assessment and surveillance of e-cigarettes and HTPs. Encountered problems and important

considerations that are crucial for the generation of reliable results are discussed in detail in this chapter. One focus was the quantitation of relevant tobacco smoke toxicants in the emissions of alternative products (2.1.1., 2.2.1., and 2.2.2.). As novel materials that are used in the products or consumables can possibly form unknown toxic substances, a method based on online-coupled pyrolysis or thermal degradation was applied for identification of thermal degradation products (2.2.3.). Further, nicotine delivery of an e-cigarette into the emissions was analyzed using a vaping machine (2.1.1.). These results were compared with observed nicotine concentrations in venous plasma upon product consumption in a clinical study (2.1.3.). Prior to the clinical study, a quantitation method for nicotine and its main metabolites in human plasma was developed and validated (2.1.2.).

3.1.1. Methods for identification and quantitation of toxicologically relevant constituents

Adaption of methods for cigarette smoke analysis

As a first step, validated and standardized methods for the analysis of cigarette smoke were adapted for HTPs. This product group was novel to the German market and methods were not implemented yet in official surveillance laboratories. Emissions were generated with a linear smoking machine for e-cigarettes, also referred to as a linear vaping machine. A linear set-up is of advantage in contrast to a rotary set-up, as heavy electrical devices such as HTPs and e-cigarettes can be connected more stably. Additionally, the emissions of individual devices can be collected and analyzed separately. While smoking machines for tobacco cigarettes enable an automated ignition and extinguishing of cigarettes, vaping machines are usually equipped with an automatic button activator. Both studied HTPs heat tobacco electrically and require activation of a button before they start the heating process.

Generated toxicant yields are not collected for a direct exposure assessment but rather for a comparison with concentrations in cigarette smoke. Thus, it is advantageous to use a standardized method for cigarette smoke generation allowing reference to a large data set. The intense puffing regime by Health Canada (HCI) was used for both HTPs. Two puffs per minute are drawn with 55 mL each, enabling more puffs during a certain timeframe. Some HTPs are programmed to turn off after some minutes like the first studied HTP (hereafter referred to as HTP 1) that turns off after six minutes. Standard methods taking only one puff per minute will draw half of the puffs than the intense method, here six instead of twelve. In consequence, HCI has been used as the only regime or together with ISO by manufacturers or independent scientists [187-193]. In the case of conventional cigarettes, burning pace is dependent on the number of puffs, as the cigarettes are mostly consumed during active puffing rather than during smoldering between puffs [194]. Consequently, cigarettes yield comparable numbers of puffs with smoking regimens with a frequency of 30 or 60 s. However, due to the intense puffing (higher interval,

higher volume) and the complete blockage of ventilation holes, yields of toxicants such as aldehydes are higher per cigarette if the HCI regimen is used compared with the ISO puffing regimen [36, 195]. This effect should be kept in mind as higher emissions of the comparator (cigarette smoke) will influence the calculated reduction of the alternative product.

Protocols for analyte trapping, sample preparation, and instrumental analysis were based on standard methods [26, 28, 29, 196] that have been validated for mainstream smoke analysis at the official control laboratory where the experiments in 2.2.1. and 2.2.2. were performed (CVUA Sigmaringen, Germany). Overall, the methods were usable for HTPs, although some adjustments had to be made. Based on published mainstream emission yields per consumable by the manufacturers [176, 192], it was estimated that concentrations of analytes in the sample solutions would be close to or even below quantitation limits. Thus, extraction volumes (for analysis of nicotine) and dilutions during sample preparation (for analysis of carbonyls) were reduced if possible. Additionally, the number of consumables puffed per sample was increased to three for TPM, water, nicotine, and carbonyl analysis, and to nine consumables per sample for determination of VOCs. Derived data were mostly in good agreement with those published by other laboratories using similar or different sample preparation methods [176, 188-190, 192, 193].

In conclusion, methods for cigarettes were applicable for HTPs as well. However, analysis might be close to detection or quantitation limits, increasing the error. To increase sensitivity, a further adaption of methods (e.g., sampling on cartridges [193], detection with tandem-mass spectrometry [197]) should be considered.

Determination of water as a problem for HTPs

As already discussed under 1.3.1. and 2.2.1., determination of water in mainstream emissions of HTP was challenging. While a correct determination of water content in product emissions has minor toxicological relevance, it is critical from a regulatory point of view. Emissions of nicotine, tar, and carbon monoxide (abbreviated TNCO) from tobacco cigarettes are regulated by the TPD Art. 3 and 4 [34]. Cigarettes must not emit more than 10 mg tar as determined applying ISO 4387 [198]. According to the referenced standard, tar is equal to nicotine-free dried particulate matter (NFDPM) which is calculated by subtraction of water and nicotine content from TPM [198]. Consequently, incorrect determination of water content can lead to systematically false values for tar with potential regulatory consequences for tobacco cigarettes. For both studied HTPs, analyzed water contents by different groups are summarized in Table 2. Experiments presented in this work and published by Li *et al.* [190] and Forster *et al.* (manufacturer of HTP 2) [176] were performed following ISO 4387. Filter holders are weighed to determine the weight gain, defined as TPM. Then, the filter holders are opened, and the glass fiber filters are placed into an Erlenmeyer flask where nicotine and water are extracted with isopropanol prior

to measurement. This extraction procedure is referred to as "external" in Table 2. Although the same product has been investigated in rooms with standardized climatizations (22°C, 60% rH, according to ISO 3402 [38]), determined water content varies between groups especially for HTP 1. The high water content in the collected product emissions, higher than the relative humidity of the laboratory atmosphere, causes the procedure to be prone to water loss. To avoid this, the manufacturer of HTP 1 has developed and used an apparatus that allows an "*in situ* extraction" of water from the unopened filter holder [192, 199]. Such a new method is helpful to understand emission composition of HTPs and to determine water content more accurately. However, additional equipment would be needed that had to become standardized and made commercially available, imposing additional costs on surveillance laboratories. Following this critique, another HTP manufacturer has proposed a modification of ISO 4387, suggesting implementation of additional wiping steps of the filter holder to improve water recovery [200]. However, this is more relevant when HTPs are regulated by their TNCO emissions similarly as tobacco cigarettes.

Table 2. Summary of total particulate matter (TPM) and water as determined by different studies for the two studied heated tobacco products (HTPs). The used extraction method (filter pad removal and external extraction in a flask or *in situ* extraction) and quantitation method (Karl-Fischer extraction or gas chromatography with thermal conductivity detection, GC/TCD) is indicated. Publications by the manufacturers of the respective devices are marked with an asterisk (*).

Device	HTP 1 (Internal heating of tobacco)				HTP 2 (External heating of tobacco)	
Source	Chapter 2.2.1. [174]	Li *et al.* [190]	Forster *et al.* [176]	Schaller *et al.** [192]	Chapter 2.2.2.	Forster *et al.** [176]
Extraction Method	External Karl-Fischer	External GC/TCD	External GC/TCD	*In situ* [199] GC/TCD	External Karl-Fisher	External GC/TCD
TPM (mg/stick)	52.6 ± 3.2	55.82 ± 1.10	48.9 ± 0.7	48.2 ± 2.4	25.4 ± 1.4	26.1 ± 1.1
Water (mg/stick)	31.7 ± 5.5	37.91 ± 0.77	25.4 ± 2.0	36.5 ± 3.1	14.7 ± 1.2	12.1 ± 1.1
Water content	60 %	68 %	52 %	76 %	58 %	46 %

Connection of differently shaped ANDS mouthpieces to the vaping machine

For investigation of e-cigarette aerosol, another problem had to be addressed: the connection of the product with the filter holder. Tobacco cigarettes and stick-shaped products can be inserted into a standardized labyrinth seal or attached with an airtight tube [32]. But design of e-cigarette is not necessarily based on the appearance of tobacco cigarettes, creating products in various shapes. The e-cigarette that was herein studied (Chapter 2.1.) had a rectangular shape, making the connection to the

filter holder with the aforementioned auxiliaries impossible. The producer of the vaping machine has developed an adaptor that was tailored to this specific e-cigarette. Although this solves the problem of connecting this particular e-cigarette model to the vaping machine, these adaptors are costly and not usable for other e-cigarettes as they must have the exact same shape, risking a potential patent infringement. It is not feasible to buy adaptors for each e-cigarette model and they therefore need to be made commercially available first. Thus, the bought adaptor was tested against a self-made adaptor, produced by heating a heat-shrinkable tubing to enclose the relevant part of the e-cigarette (Figure 9).

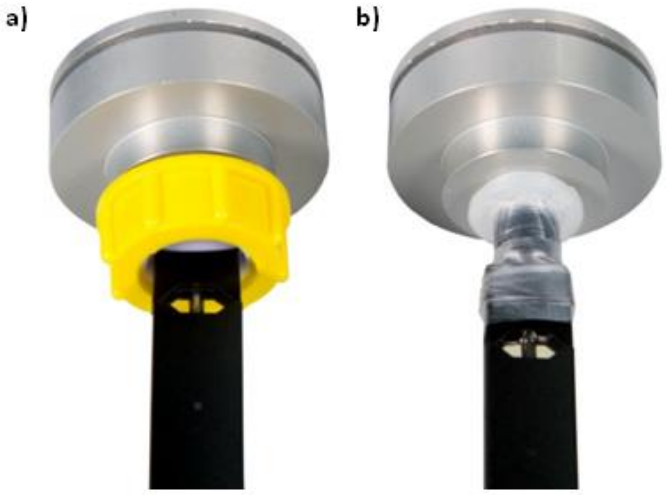

Figure 9. Pictures of the rectangular pod e-cigarette connected to the filter holder using **a)** the bought adaptor developed by the vaping machine producer, and **b)** the self-made adaptor assisted with parafilm. Figure is downscaled from the Supplementary Material of [171], presented in Annex I of this thesis.

TPM yield was compared when using both adaptors (2.1.1.). Although there were slight differences, TPM yield was sufficiently comparable to conclude that the heat-shrinkable tubing can be a suitable and cost-effective alternative to commercial adaptors. Other materials, for example 3D-printed adaptors, could be considered when the adopters can be made air-tight without blocking holes that are necessary for ventilation.

Identification of emerging risks from novel materials used in ANDS

A prerequisite for harm reduction by ANDS is that exposure to harmful substances is reduced. This does not only include toxicants that are established for tobacco smoke, but also substances that have a new relevance. These can be formed or released into HTP or e-cigarette aerosol due to new ingredients, the specific composition, other thermal processes, or novel materials that are not common for cigarettes. Before these substances can be quantified to be integrated into the risk assessment of the products, they need to be identified first using untargeted screening. Samples can either be collected and analyzed offline or sample generation and measurement instruments can be coupled online. Depending on the sampling method and analytical technique, recovered and identified compounds can differ. For

example, Bentley *et al.* have applied two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GCxGC-TOF) and liquid chromatography coupled with high-resolution accurate mass spectrometry (LC-HRAM-MS) to identify and semi-quantify chemicals in collected particulate and gas vapor phase of HTP emissions [177]. Rawlinson *et al.* have collected e-cigarette aerosol on sorbent tubes and characterized components using gas chromatography with time-of-flight mass spectrometric detection equipped with a thermal desorption unit (TD-GC-TOF) [201].

Further, fate of tobacco or other used materials under heat can be studied separately. Li *et al.* have studied degradation of HTP tobacco at operation temperatures using two-dimensional gas chromatography with mass spectrometric detection (GCxGC-MS) online-coupled to an analytical pyrolyzer [190]. Online-coupled pyrolysis has been demonstrated to be a useful tool to screen for degradation products of tobacco and tobacco additives [181, 182]. Consumables for HTP 1 contain different filters, one of them made of polylactic acid (PLA). Visible changes of material color and integrity after consumption suggest that the filter comes into contact with heat. To study formation of thermal-degradation products, online-coupled pyrolysis gas chromatography – mass spectrometry (Pyr-GC/MS) was used as described in **2.2.3.** Pyrolysis results do not necessarily reflect actual substance generation and their quantities in emissions, as for example interactions with other compounds or materials are not considered. However, pyrolysis is useful to identify candidates for further investigation. An advantage of online coupling is a reduced loss of analytes. Davis *et al.* have used an offline method to analyze heated PLA filters of HTP 1 using headspace gas chromatography with mass spectrometric detection (HS-GC/MS) [183]. While with online-coupled Pyr-GC/MS toxic volatile compound acetaldehyde was detected (**2.2.3.**), it was not detected using the offline method [183].

A comprehensive chemical assessment cannot be performed based on only one analytical method. Unknowns can only be identified if they are extractable with the applied sample generation and preparation protocol and are detectable with the used instrument and detector. For example, gas chromatography can only process substances that can be vaporized intact under the applied conditions (i.e., temperature, pressure), mass spectrometers detect only substances that are ionized by the selected ion source, and any sample handling step can cause the loss of certain analytes. Thus, several methods for unknown identification should be applied complementary. Online-coupled Pyr-GC/MS has been demonstrated to be a useful and complementary tool for identification of generated substances from new materials.

3.1.2. Prediction of nicotine delivery with analytical methods

Using the same pod e-cigarette, nicotine was quantified in mainstream emissions generated with a vaping machine (**2.1.1.**) and nicotine delivery into the blood upon actual consumption was assessed in a clinical study (**2.1.3.**). Further, a product modification with influence on vapor generation was

followed-up with both studies. This allows to draw conclusions on the relationship between the data generated with both methods.

Gravimetric determination of nicotine content in the emissions

A model predicting the nicotine flux (defined by the authors as aerosolized nicotine mass per time) based on e-cigarette design features, power settings, and liquid composition was established by Talih *et al.* [119]. Dynamics are described by the authors for the vaporization rates of liquid components with different volatilities (PG > nicotine > VG; from higher to lower volatility) based on Raoult's Law: while in the beginning of the puff (termed transient phase), the more volatile component is vaporized at a higher rate and depletes in the liquid in the vicinity of the heating coil, a steady-state phase is reached after some time [119]. In this steady-state phase, vaporization rate of the less volatile component (VG) approximates the decreased vaporization rate of the more volatile component (PG) [119]. A higher power setting promotes a faster attainment of steady-state, while this steady-state is not reached if the power setting is too low (2 W in this case) [119]. The authors have demonstrated mathematically and experimentally that nicotine flux is dependent on the amount of vaporized liquid and that nicotine concentration in the emissions over the complete puff equals the nicotine concentration in the liquid [119]. Consequently, nicotine flux can be calculated when the TPM or the respective liquid consumption (that are "to negligible error equal") is multiplied by the nicotine concentration on the liquid [119].

While the model by Talih *et al.* assumed a sufficiently rapid supply of liquid to the coil [119], the performance of the initially used wick material in the investigated e-cigarette has been demonstrated to be potentially rate-limiting (**2.1.1.**). Thus, the two observations "TPM and liquid consumption are (almost) equal" and "nicotine concentration in emissions can be calculated using TPM or liquid consumption and nicotine concentration in liquid" have been tested and were confirmed with the herein studied pod e-cigarette (data are presented in **Annex I**) [171].

This knowledge was used in **2.1.3.** to **a)** determine nicotine delivery per two machine generated puffs without the need for a sensitive analytical instrument, and **b)** to compare delivered nicotine doses calculated via the liquid that was consumed by the study participant or in the vaping machine experiment. This means that nicotine delivery into the aerosol can be determined using a precision scale as the only analytical instrument. Given a sufficient precision of the scale, determination of low nicotine deliveries is not limited by the sensitivity of the instrument that would have been used for chemical analysis. Additionally, gravimetric determination of nicotine yield could save time and costs during emission studies. Further, the liquid that is consumed by a participant of a clinical study or by a consumer in real-life can be assessed and a nicotine dose can be calculated. Even though this approach will not result in information on nicotine kinetics (e.g., C_{max} or t_{max}), it can be easily performed without blood sampling and analysis. It can give an estimation of the administered nicotine dose at stage of

consumption. It is further possible to estimate a usual daily nicotine dose if the volume of the usually consumed liquid per day and nicotine concentration in the liquid is known. In addition, calculated nicotine dose can be a helpful tool to compare results from vaping machine experiments and clinical studies, as performed below.

Relationship between delivered amount of nicotine in vaping machine experiments and upon product use by humans

In 2.1.1., emissions of two pod variants of the same e-cigarette with the same nicotine concentration in the liquid were compared. The initial pod variant contained a wick made from a material that did not swell enough to sufficiently supply the coil with fresh liquid. Accordingly, vapor generation was lower compared with the modified pod variant containing a wick made from a material with a better performance. However, these differences did not lead to different nicotine concentrations in the participant's blood in the clinical follow-up study (2.1.3.). Consumed masses of the liquid were the same in both study groups, resulting in the same calculated nicotine doses. Table 3 displays calculated nicotine doses for different vaping experiments from both study parts together with relevant data and parameters. In 2.1.1., nicotine yields in emissions were also analyzed using GC/FID, resulting in analyzed nicotine yields per puff of 23 µg, 61 µg, and 72 µg for the initial EU, the modified EU, and the US variant, respectively. This is in agreement with the calculated doses.

Two conclusions can be drawn from the evaluation of the results from both studies. The first conclusion is that the liquid consumption in the clinical part was correctly predicted with the vaping machine experiments when the same number of puffs was used (2.1.3.). Puff volume of 55 mL was applied in the vaping machine experiments according to standardized puffing regimen. Talih *et al.* argued that the puff flow rate, meaning a changing puff volume with a fixed puff duration, does not have an influence on TPM and nicotine yield [118, 119]. Instead, the amount of generated aerosol is dependent on the heating time that usually equals the puff duration [118, 119]. Thus, it is in line with existing literature and mathematical models that given a fixed puff duration, TPM and nicotine yields can be predicted. This opens the question why the vaping machine experiments in 2.1.1. failed to predict the outcome of the clinical study. While prediction models assume the absence of a rate-limitation by the wick [119], the wick material that has been used in the initial variant was demonstrated to be a limiting factor, as already discussed. In the first study, a total of 160 puffs was drawn in sets of 20 puffs from each analyzed pod with a cool-down time of ten minutes in between sets. This allowed difference in the wick design to come into effect. It is assumed that ten puffs are not enough for the initial wick to show an inferior supply of liquid to the coil. In the clinical study, each participant was provided with a fresh pod due to obvious hygienic reasons and took only 10 puffs before the pod was discarded. It is possible that in a longer study with more puffs taken from each pod, the differences in wick performance would come

into effect in the clinical setting as well. In this case, it is presumed that the nicotine delivery by the initial pod variant became worse. This might also occur if the US version that contains the same wick material was studied for a longer number of puffs.

Table 3. Nicotine dose per puff was calculated for puff clusters that were drawn by a vaping machine or by humans in a clinical study from different variants of the same pod e-cigarette brand. Data from both study parts (2.1.1. and 2.1.3.) were integrated. Either TPM or liquid consumption represent vapor amount. Rounded mean values have been used and numbers of repetition differed between columns.

Study part	2.1.1. [171]			2.1.3. [173]			
Puffs drawn by	Vaping machine			Vaping machine		Human	
Pod variant	Initial EU variant	Modified EU variant	US variant	Initial EU variant	Modified EU variant	Initial EU variant	Modified EU variant
Liquid nicotine concentration	15 µg/mg	15 µg/mg	50 µg/mg‡	15 µg/mg	15 µg/mg	15 µg/mg	15 µg/mg
Puff duration	3 s	3 s	3 s	3 s	3 s	3 s*	3 s*
Puff volume	55 mL	55 mL	55 mL	55 mL	55 mL	n.d.	n.d.
Puff number	**160**	**160**	**160**	**10**	**10**	**10**	**10**
Mean TPM or liquid consumption	256 mg (TPM)	592 mg (TPM)	224 mg (TPM)	35 mg (liquid c.)	31 mg (liquid c.)	31 mg (liquid c.)	32 mg (liquid c.)
Calculated nicotine dose	3 840 µg	8 880 µg	11 200 µg	525 µg	465 µg	465 µg	480 µg
Calculated nicotine dose per puff	**24 µg**	**56 µg**	**70 µg**	**53 µg**	**47 µg**	**47 µg**	**48 µg**

‡ Liquid nicotine concentration of US variant was not assessed in this work. The labelled concentration of 50 µg/mg (5 %) was used. * Participants were instructed to take puffs with a duration of 3 s following an acoustic signal. n.d.: Puff volume was not determined in the clinical study.

In consequence, it seems possible to predict the nicotine dose upon actual consumption with a mathematical model [119] or with a vaping machine (as performed in 2.1.3.) provided that puff topography, especially puff duration, is known or estimated. Puff duration has been demonstrated to depend on factors such as user experience [104, 105], liquid flavors [106, 108], and nicotine strength [111]. However, uncommon design features such as a wick with an inferior performance complicate predictability. It is possible that other e-cigarettes and emerging ANDS offer such problems. This should be considered when results from vaping machine experiments are extrapolated to human consumption.

3.2. Assessment of health risks

Assessment of health risks is best supported by different types of data, from exposure data to information on health-related outcomes in humans. However, data on new products are usually scarce and an initial assessment has to rely on information that can be acquired fast and readily. Quantities of toxicologically relevant constituents in the emissions can be rapidly determined and can be used to estimate relative exposure in comparison with other products. One aim of this work was the assessment of selected HPHCs in mainstream emissions in HTPs and e-cigarettes. Emission testing of products was focused on carbonyl compounds for e-cigarettes and on carbonyl compounds and VOCs for HTPs. As already discussed before, VOCs such as 1,3-butadiene and carbonyl compounds such as acetaldehyde and formaldehyde are of special relevance for the carcinogenicity and respiratory toxicity of tobacco smoke [20]. Based on their cancer risk indices, aldehydes and small organics were calculated to contribute about 62% to cancer risk index of tobacco cigarette smoke [20]. For e-cigarettes, carbonyl compounds are the most relevant group of tobacco smoke toxicants being formed from main components PG and VG under heating conditions [126-129]. In this chapter, generated data on toxicant emissions are compared and discussed in the context of biomarker studies in the literature. Implications for individual health risks are extracted from emission data, followed by an introduction of important public health considerations.

3.2.1. Toxicologically relevant constituents in emissions of e-cigarettes and HTPs

In the emissions of both studied HTPs, content of 1,3-butadiene and other tested VOCs was reduced by over 96% compared with tobacco smoke (**2.2.1.** and **2.2.2.**). Reduction of carbonyls was less pronounced. HTP 1 emitted approximately 180 µg acetaldehyde per consumable (**2.2.1.**). Although this concentration was reduced compared with tobacco cigarette smoke (80-90%), this product still represents a relevant source for this carcinogen (Carc. 1B according to CLP regulation [132, 133]). Further, the actual exposure to acetaldehyde is likely to be higher. Firstly, only 12 of possible 14 puffs were taken during the assessment. Secondly, nicotine emission was with 1.1 mg per consumable (with 12 puffs) in the range of cigarettes with low nicotine yield when using the HCI puffing regime [175]. The reduced nicotine delivery of low tar cigarettes has been shown to lead to “compensatory puffing” in consumers who aim to extract more nicotine [91-93]. Therefore, for better comparison between products considering potential compensation effects, acetaldehyde emissions can be expressed as 163 µg per mg nicotine. Reporting of toxicant levels per mg nicotine has been recommended by WHO TobReg [18]. Emission of acetaldehyde by HTP 2 was 91 µg and lower compared with HTP 1 (**2.2.2.**). However, nicotine yield was with 0.32 mg also reduced. Accordingly, acetaldehyde emission per mg

nicotine was with 284 µg even higher compared with HTP 1. Formaldehyde emissions per mg nicotine were 4.8 µg for HTP 1 and 8.4 µg for HTP 2 (**2.2.1.** and **2.2.2.**).

Counts *et al.* have published a large set of emission data from 50 international cigarette types with different nicotine strengths including two reference cigarettes generated with three different smoking regimens [175]. Emissions generated with HCI regime were extracted from the publication and used to calculate ratios of acetaldehyde and formaldehyde to nicotine (in µg carbonyl compound/mg nicotine) [175]. Acetaldehyde to nicotine ratio ranged from 450 to 1000 µg/mg and formaldehyde to nicotine ratio ranged from approximately 20 to 90 µg/mg (based on data from [175]). When put into context with nicotine yield, reductions of carbonyl emissions by the investigated HTPs can be far below the previously mentioned 80%, if compared with some cigarettes. Reduction of carbonyl yield per mg nicotine by HTP 2 was only 40 – 60 % compared to the lower bound of the above-mentioned calculation using cigarette smoke yields. Thus, more research is needed to follow up compensatory effects in context of HTP consumption.

Carbonyl emission by e-cigarettes is influenced by factors such as temperature, power to coil surface ratio, and produced aerosol as discussed under **1.2.2** [97, 129, 137]. One important design feature of the herein investigated pod e-cigarette is the low power vaporization leading to inconspicuous plumes of exhaled aerosol. This has given rise to internet challenges in the United States, in which young people filmed themselves secretly using the product at places where e-cigarette consumption was prohibited, referred to as “stealth vaping” [202, 203]. The low vaporization power suggests a low thermal degradation of liquid components and thus a low formation of toxic carbonyl compounds. In fact, acetaldehyde emission per mg nicotine was 3 µg and 0.2 µg determined drawing 160 puffs from the initial and the modified version, respectively. Formaldehyde emissions per mg nicotine were 5 µg and 0.2 µg (**2.1.1.**). Considering the wide range of measured carbonyl compound concentrations in e-cigarette aerosol (see **1.2.2.**), levels found in this study can be considered as rather low.

Emission data are only the first step to assess exposure and should be evaluated in context of clinical data. Biomarkers of exposure (BoE), e.g., monohydroxy-3-butenyl mercapturic acid (MHBMA) for 1,3-butadiene [204], are analyzed in clinical trials or cross-sectional studies. Several studies have shown a significant reduction of BoE after switching to HTPs or e-cigarettes, as summarized in a recent systematic review by Akiyama *et al.* [185] and a meta-analysis by Drovandi *et al.* [186]. However, eight of twelve of the BoE that were assessed for HTPs, including MHBMA, were significantly reduced when compared with cigarette smoking but not when compared with smoking abstinence [186]. Biomarkers for acetaldehyde or formaldehyde were not assessed in clinical trials for HTPs [185, 186]. Akiyama *et al.* have assessed BoE studies that compare HTPs and e-cigarettes with tobacco cigarettes and abstinence. They have concluded that there was some but no major or consistent evidence for a higher reduction

of BoE from e-cigarettes than from HTPs [185]. However, there was no study directly comparing both products with each other [185].

3.2.2. Health risks for individual consumers

Health risk is composed of the hazard (harmfulness of the constituents) and the exposure (concentrations in emissions combined with user behavior). According to carbonyl and VOC levels analyzed in product emissions, health risks posed by products can be ranked; The most toxic product remains the conventional cigarette, followed by discussed HTPs, and discussed e-cigarettes. No use of either tobacco cigarettes or ANDS poses the lowest risk to regular, former, and non-smokers. This is in agreement with the existing literature, e.g., using mathematical models to calculate lifetime cancer risks [168]. In terms of harm reduction, an addicted smoker would benefit from a complete switch to any of the discussed products if continuation of tobacco cigarettes would be the only alternative. Still, first recommendations for smokers should be other cessation aids adhering to professional guidelines [44]. Individual choice of product could be made according to the reduction of exposure but should also consider product liking and potential adherence. The latter is important to avoid dual use of the alternative product and conventional cigarettes. Biomarker studies in dual users of cigarettes and e-cigarettes have shown a higher exposure to toxicants compared to cigarette-only smokers indicating an increased health risk [167]. Anticipated risk reduction for the individual consumer is not necessarily numbered the same as the exposure reduction but is most likely not zero. However, the ultimate goal should still be a complete cessation of cigarette and ANDS use. Even if products did not emit any toxic compounds other than nicotine, maintenance of addiction, physical as well as conditioned, is already a severe health problem. Further, negative cardiovascular effects can be caused by a high nicotine delivery [205, 206] and a negative impact on the respiratory system is possible [207, 208].

Emerging category of heated tobacco products

The substance with the highest contribution to cancer risk, 1,3-butadiene [20], was reduced more than 99 % in the emissions of both HTPs regardless of the amount of emitted nicotine. This should be kept in mind if new risks are discovered. These must outweigh the reduction of the other highly toxic substances to counteract the reduction of health risk. Nevertheless, new harmful compounds need to be assessed and monitored. Discussed HTPs still pose a relevant health risk to the consumer as for instance they emit notable levels of carcinogenic carbonyl compounds. Quantitative risk assessment studies by independent researchers have estimated cancer risk on the basis of selected carcinogens that are relevant for cigarette smoke (further discussed under 3.3.2) [168, 169]. They came to the preliminary conclusion that HTPs still impose a health risk that is however substantially lower than that of cigarette smoking [168, 169]. Notably, emissions of hazardous compounds are as of yet unclear for new products that will be launched in the future, especially knock-off products. Risks could be lower or higher

compared with the products discussed herein. Thus, consumers would benefit from a regulation of HTPs regarding maximum emission levels as discussed below under 3.3.2.

3.2.3. Public health considerations

Besides the potential influence on the health of the individual consumer (decreasing or increasing health risk), ANDS have an impact on the health of the population (potentially decreasing or increasing net public health risk). For instance, some ANDS may help addicted smokers to reduce their individual risks but will still cause an increased risks for public health when they are predominantly used by non-smokers. To draw conclusions on public health effects, a row of different data is required as pictured in Figure 10. In this thesis, only questions are addressed that could be sorted into the first black box on product characteristics. As many more information needed to be obtained and integrated, it would be scientifically unsound to draw conclusions on the studied products' population health effects solely based on the presented data. Thus, important public health considerations are only briefly introduced here.

Figure 10. Simplified illustration of information that is needed to evaluate public health effects of alternative nicotine delivery systems (ANDS) divided into three "black boxes", i.e., research areas with some exemplary research questions.

ANDS can have a negative impact to population health if they lead to an overall increased exposure to harm over time. This includes non-smokers and former smokers who initiate ANDS use, at worst followed by initiation of cigarette smoking. The latter is usually referred to as the "gateway effect" [209]. Cigarette consumption might also increase indirectly through a possible renormalization of cigarette-like nicotine consumption [87, 210]. Advertisement and especially penetration of the market most importantly among peer groups play a major role in use initiation [211-213]. The financial interest of manufacturers of cigarettes and related products in maintaining and gaining a high number of consumers stands in direct contrast to public health goals to reduce consumer numbers [84]. Most smokers initiate cigarette use at young age [214, 215]. Someone who starts smoking at a young age might have a whole life of exposure to cigarette smoke ahead if future cessation attempts remain

unsuccessful. Further, nicotine has been shown to interfere with neural development with a detrimental impact on the developing brain, as reviewed by Dwyer *et al.* [216].

On the other hand, youth protection and harm reduction for addicted smokers do not necessarily contradict each other, as argued by Fairchild *et al.* [87]. Further, ethical defensibility of favoring the minimization of young people's risks over those of older smokers was questioned [217]. Beyond this brief introduction into potential public health effects of ANDS, this topic is far more complex. A framework that can be used to evaluate the population health impact of e-cigarettes and related products has been proposed by Levy *et al.*, discussing potential pathways for different consumer groups [170].

Taken together, risk communicators and regulators are forced to walk a tightrope to find the right balance between offering harm reduction alternatives for addicted smokers and protecting the rest of the population.

3.3. Regulation of ANDS

Regulation of ANDS is challenging. Protection of the population and especially vulnerable groups from avoidable risks is one important yet difficult task. Products should not be appealing to non-smokers and should not induce addiction. Product uptake by naïve users might lead to tobacco dependence and should be prevented by regulatory efforts. If a harm reduction strategy is followed, it needs to be considered that they might become less effective depending on regulation. Tobacco regulation has not been studied in this thesis and only selected parts are discussed herein. Thus, regulatory considerations are not discussed in their entirety. For example, how strictly use of new products should be regulated and whether a harm reduction strategy is advisable is not addressed. If ANDS are allowed on the market to be used to replace combustible cigarettes, they should deliver the lowest possible amount of toxicologically relevant compounds. Then, a regulatory limitation of the exposure to hazardous compounds is suggested. However, it first needed to be determined which substances are relevant for emerging products and what limits are feasible.

Another major problem with regulation of ANDS is that definitions that determine the scope of application are quite narrow and quickly outdated. As a consequence, new products enter the market that deliver nicotine but do not fall within the scope of the TPD as existing product categories do not apply. One example are all-white nicotine pouches that look like pouched snus (a type of oral tobacco product), are used like pouched snus, and contain nicotine in considerable amounts [83]. Nevertheless, these products do not contain tobacco, are therefore no oral tobacco products, and do not fall within the scope of the TPD [34].

3.3.1. Regulation of e-cigarettes

As discussed above, e-cigarettes have the potential to be a less harmful alternative for cigarette smokers but also bear the risk to maintain or induce nicotine consumption and addiction in smokers or naïve users. In the United States, e-cigarette use in adolescents has become a serious problem in recent years. E-cigarette use among US high school students peaked in 2019 with a prevalence of 27.5% [218]. In 2020, 22.5% of US high school students used e-cigarettes daily [219]. In comparison, 14.5% of adolescents (12 to 17 years old) in Germany had ever tried e-cigarettes in 2019 [220]. Only 4.1% had reported to have used e-cigarettes in the past 30 days [220]. This raises the question of what has happened in the US and how to prevent this harmful trend to enter other countries. Most popular types of e-cigarettes in 2020 among US high and middle school students were pod e-cigarettes with 48.5% and 41.3%, respectively [221]. Pod e-cigarettes are characterized by their closed system enabling easy use, meaning that the pods are ready-to-use, already containing heating element and complete liquid. A pod e-cigarette brand that has drawn much attention is JUUL. JUUL pods were available in the US

initially with only one nicotine strength of 5% (w/w), and later also with 3% (w/w) [222]. These weight-based concentrations translate into volume-based concentrations of 35 and 58 mg/mL (with a density of 1.16 g/cm³ [171]). A study in JUUL-using high school students has revealed that the majority of participants did not appraise the nicotine content of the 5% JUUL pods as high [222]. Nicotine delivery by the highest nicotine version of JUUL (5% nicotine) has been demonstrated to be comparable to conventional cigarettes in experienced users [116]. Unlike with open systems, options for personalization of liquid composition and vaporization power are not given by pod e-cigarettes beyond the variety of available pods. Consequently, users could only select between two high nicotine strengths. However, consumers could manipulate or refill pods or use knock-off pods with other compositions. This increases problems and uncertainties for product regulation and surveillance.

Already in 2017, JUUL has reached about half of the US e-cigarette market share [223]. The brand was advertised heavily on social media including affiliate marketing [223]. Reasons for product liking by high school students were the nicotine buzz associated with the high nicotine delivery, liking of the flavors, and use by peers [212]. Frequency of JUUL use was associated with liking of nicotine effects [212]. Among college students, the main reasons for JUUL use initiation were curiosity, use by friends, the absence of bad smell, ease of product use, and liking of flavors [213]. For use continuation, ease of use was the main reason accompanied by other reasons related to the e-cigarette design, flavors, but also related to relaxation during stress [213]. Interestingly, approximately one third of JUUL users participating in a study by Ickes *et al.* did not define themselves as e-cigarette users [213]. In consequence of the high rise in adolescent e-cigarette users, pod e-cigarettes with flavors other than tobacco and menthol have been subject of an enforcement policy by FDA early 2020 [224].

JUUL was launched in Europe at the end of 2018 and has been studied in this thesis (**2.1.1.** and **2.1.3.**). A feared high uptake of JUUL and other e-cigarettes by adolescents did not become apparent in Germany [220, 225]. To understand this, differences between JUUL e-cigarette in the US and JUUL e-cigarette in Germany need to be discussed. The most apparent difference is the lower nicotine content in the European version. Liquids for e-cigarettes are regulated by the TPD with a maximum content of 20 mg/mL (Art. 20) [34]. The European version of JUUL is available in two nicotine strengths, 9.0 and 17.7 mg/mL (**2.1.1.**). Nicotine delivery in the acute phase by the 17.7 mg/mL version was much lower in experienced e-cigarette users compared with cigarette smoking by routine smokers (**2.1.3.**). A rapid increase of blood nicotine levels in the first minutes is associated with induction of addiction [51, 52]. Besides the lower nicotine delivery, differences in marketing have most likely played a role in the low uptake of the product. The manufacturer of JUUL has not used the same social media marketing strategy in Germany [226]. Importance of marketing and regulation thereof has been acknowledged by German regulators. Wide-ranging bans for marketing of tobacco products and e-cigarettes, including social media marketing, have been passed in Germany in 2020 (2. TabakerzGÄndG [227], TMGuaÄndG [228]).

Outdoor advertising bans will gradually enter into force by January 2024 depending on the product type [227].

ANDS with a high nicotine delivery can be regarded as a coin with two sides, one side is the possibility to be a gateway product into smoking and the other side is their potential for harm reduction in smokers. ANDS with a high nicotine delivery in the acute phase could induce addiction in experimenting users [51, 52]. Cigarette smokers might require a certain nicotine delivery for adhering to their alternative product [105, 229, 230]. A Cochrane review on e-cigarettes in smoking cessation has found moderate-certainty evidence for a benefit of nicotine containing e-cigarettes compared with nicotine-free products for at least six months [231]. Further, as discussed under **3.2.1.**, a high ratio of exposure to hazardous substances to delivered nicotine is unfavorable. Compensatory puffing has been shown in e-cigarette users when a liquid with a lower nicotine content was used [232]. The authors have used the observed puffing parameters in a vaping machine study to simulate carbonyl formation and confirmed the hypothesized higher carbonyl formation with the compensatory puffing behavior [233].

In summary, nicotine delivery is a critical parameter for the product's potential risk or potential benefit, making it vital for regulators. However, nicotine delivery is not only dependent on nicotine concentration in the liquid, but also on other influential factors such as puff duration, liquid composition, power settings, and even the wick material used [118, 119, 171]. As this complicates regulation of nicotine delivery by open system e-cigarettes, Shihadeh and Eissenberg have proposed to regulate the "nicotine flux" (delivered nicotine per puff second) taking into account the product characteristics and puffing behavior [234, 235]. The range of nicotine fluxes by one device is computed using a model feeding in the relevant product characteristics (e.g., liquid nicotine concentrations, voltage, vaporization efficiency) and all possible and plausible puffing topographies. Consequently, products with an unfavorable combination of design characteristics that would lead to an ineffectively low or unsafely high nicotine flux at expectable puffing topographies could be prevented [234, 235]. Since the market is under constant development, also previously neglectable product design characteristics may appear on the list of relevant features, as for example the wick material (discussed under **2.1.1.**, **2.1.3.**, and **3.1.2.**). Thus, models used for prediction have to be updated regularly.

Nicotine delivery to the consumer by closed system e-cigarettes is easier to regulate by restricting the liquid nicotine concentration. Although this only affects a limited number of products, it still might be of relevance. Restrictions for pod e-cigarettes in particular have been introduced due their appeal for young people [224]. However, it is debated that such easy-to-use e-cigarettes might be appealing for highly dependent harder-to-treat smokers as well, though more research is needed to better understand this issue [236].

It should be noted that "less harmful" does not mean without any harm. E-cigarettes still cause exposure to harmful substances and pose an avoidable health risk. They are not healthy or ideal products and are certainly the worse alternative compared with complete nicotine use cessation. However, continuation of cigarette smoking is the worst option.

3.3.2. Regulation of HTPs and other novel products

The TPD defines the product category "novel tobacco products" as tobacco products that do not fall in certain categories (cigarettes, roll-your-own tobacco, pipe tobacco, waterpipe tobacco, cigars, cigarillos, chewing tobacco, nasal tobacco, tobacco for oral use) and were placed on the market after 19th May 2014 [34]. These products require notification to and possibly authorization by member states and should comply with the requirements of the TPD [34]. Some European countries regulate HTPs as novel tobacco products, other countries have sorted these products in one of the existing categories such as e-cigarettes or smokeless tobacco [237]. The applied product category can have a huge influence on the product's regulation, potentially leading to circumvention of bans for indoor use or characterizing flavors.

When HTPs are authorized on the market, the next question of how emission levels could be regulated arises. A regulation of TNCO levels as for tobacco cigarettes would not make much sense. Even for cigarettes, regulation of TNCO is disputed [19] and it is less appropriate for regulation HTPs. Tar resembles the particulate mass that was trapped on the glass-fiber filter deducting the amount of water and nicotine [198]. As discussed under **2.2.1.** and **3.1.1.**, this remaining mass on the filter rather resembles the high proportion of humectants in HTP emissions than the amount of generated cigarette smoke. Further, relevant carcinogens in the context of HTP emissions are volatile. Emission limits of priority analytes, for example listed by WHO TobReg [18], would be more applicable for HTPs. However, these priority lists are based on knowledge on cigarette smoke and need to be updated regularly based on hazardous components that become relevant in the emissions of new products. This is especially challenging when the product category is as heterogeneous as HTPs are.

Approaches that do not rely on a predetermined list of compounds would be more applicable. Fowles and Dybing have applied cancer potency factors (CPFs) and reference exposure levels (RELs) to calculate contribution of different constituents on risks of tobacco smoking based on machine smoking yields [20]. Cancer risk indices and non-cancer risk indices for certain target organs were calculated. Although the authors argued that the derived risk indices do not reflect actual cancer risks, the method is useful for a relative assessment [20]. Stephens has applied a similar approach using CPFs to calculate lifetime cancer risks and a relative cancer potency based on the exposure scenario (e.g., inhalation of ambient air, e-cigarette aerosol, HTP aerosol, or tobacco smoke) [168]. Slob *et al.* have advanced the approach, calculating changes in cumulative exposure (CCE) based on relative potency factors (RPFs) derived from

benchmark doses (BMDs) [169]. This way, they have transformed the different dose-response relationships of a mixture of chemicals into the dose-response relationship of only one single substance, the reference compound. Briefly summarized, they have estimated RPFs in relation to the selected reference compound, 1,3-butadiene, based on BMDs for different chemicals found in the emissions of both cigarettes and HTPs. RPFs were multiplied by the respective concentrations in mainstream emissions as determined by published analytical studies. CCE was calculated as the ratio of the added results for HTPs and cigarettes [169]. It should be noted that such approaches neglect potentially important factors, e.g., particle effects [168].

The mentioned approaches were developed for risk assessment of the products and rely on previously determined quantities of established compounds. However, such a similar framework might be useful for regulation of heterogeneous and developing product groups (i.e., HTPs or even ANDS in general). For this, such an approach needed to be adapted to be used in combination with an untargeted analytical assessment (discussed under 3.1.1.). All identified compounds that contribute to the products toxicity would factor into the calculation of a "risk score". Regulators could set upper thresholds for the derived "risk scores" that must not be exceeded by products to be allowed on the market. The untargeted analytical assessment should be a framework of different screening methods to be able to capture different compound groups, e.g., volatiles, non-volatile organic compounds, and metals. The whole method including aerosol generation and sampling should be standardized. An exact quantitation is not necessary as the scores would be used for a regulatory purpose rather than to estimate actual risks. Thus, a semi-quantitation, relative to nicotine or an internal standard, would be more applicable and feasible than quantitation of each identified product. Such an approach would help to regulate ANDS based on their product emissions without the need to define the relevant compounds first. These thresholds could even be successively lowered analogously to strategies proposed for tobacco cigarettes.

4. Conclusion and Outlook

Reliable quantitation for surveillance and regulatory purposes requires standardization of analytical methods. As discussed, some standard methods for cigarette smoke analysis can be easily adapted for determination of HTP emissions, but not all (e.g., for water). Standardization of methods for HTPs by international standardization bodies is already in progress. For instance, CORESTA has started a HTP task force in 2019 [238], ISO has established a working group for tobacco heating systems (ISO/TC 126/WG 22) [239]. However, development, validation, and standardization of methods for HTPs is complicated by the absence of reference materials. It would be impracticable to supply reference consumables for every different device. A more feasible option would be a reference combination of device and consumable that is not meant for consumption and is produced over a sufficiently long time period without variations in design. Due to the complexity of the product group, only one such reference product will hardly represent the expectable variety in emission composition. Consequently, reference devices and consumables with different tobacco heating mechanisms would be needed.

The necessity for development and standardization of quantitation methods follows the need to know the quantities. Determination of quantities of hazardous compounds in HTP emissions is important to perform a risk assessment. However, maximum limits are not in force. In the chapter above (3.3.2.), a different regulatory framework was proposed that does not require quantitation of constituents.

Steps for development and establishment of the herein proposed regulatory framework would be:

- Development and standardization of the untargeted screening methods, including the methodology for aerosol generation. Methods need to be capable to identify compound groups with different physicochemical properties and should be feasible for governmental laboratories. The validity of the semi-quantitation approach should be verified by actual quantitation of all toxicologically relevant constituents by a group of “test” or “training” products.
- The most sensible framework for calculation of “risk scores” based on quantitative risk assessment approaches needs to be established by experienced toxicologists. It should be kept in mind that the derived risk scores do not need to be meaningful for assessment of actual risk or exposure. In consequence, scoring factors for individual compounds may also take account of variables such as differences in the instrument response.
- Establishment of a database of a multitude of possible constituents would be time and work consuming but necessary. However, such a database could be valuable in the future for products that are not even thought of yet. As such a database should contain the most likely and relevant possible compounds to be found in HTP emissions, some newly identified

compounds might not be included. A guideline to estimate auxiliary scoring factors for such compounds needed to be provided.

- Finally, a market screening of risk scores of available HTPs in combination with already established risk assessment tools should inform regulators to help them set appropriate upper limits.

Further research is needed regarding methods to study e-cigarettes as well. Standardization efforts have already produced a selection of methods and reference materials that can be used for product surveillance [240-242]. Method development continues, for instance, clarifying whether, and if which, adaptions need to be made to reflect differences in e-cigarette types and consumption of them [99, 100]. New analytes (e.g., aroma compounds) are relevant for e-cigarettes in comparison to tobacco cigarettes requiring dedicated quantitation methods. Additional information on the liquid and aerosol chemistry is needed. For risk assessment, it is important to gain knowledge about product characteristics such as nicotine delivery to the consumer in a timely manner. The predictability of such factors by simpler analytical methods like machine vaping needs to be assessed. In this work, vaping machine nicotine yields were related to blood nicotine concentrations after human consumption following a pre-directed puffing regimen. The consumed amount of liquid was the same with both methods, when the same parameters were applied, suggesting a good predictability. These results need to be linked with the next step, nicotine delivery after *ad libitum* use of the same product, a setting that is closer to real-world use. In the following, it will be elicited whether machine vaping studies can predict actual nicotine delivery under near real-world scenarios, an important factor for addictiveness and craving reduction by the product, and under which conditions these predictions can be made. Using pod-type e-cigarettes for such evaluations has the advantage that the product settings are fixed and are thus the same for all steps, provided that the manufacturer does not change the product characteristics. Further technical parameters (e.g., the wick material) or characteristics of the resulting emissions (e.g., presence of other chemicals) might be detected to be relevant for uptake of nicotine and should be included in prediction models.

Besides the methodological questions, data were generated to enable an initial science-based risk assessment of the investigated products. In conclusion, the studied e-cigarette and HTPs seem to lead to a lower exposure to hazardous, partly carcinogenic, compounds in comparison to tobacco cigarettes. Current knowledge suggests that smokers, who are reluctant to quit nicotine use, would reduce their health risks when switching from cigarette smoking to use of e-cigarettes or HTPs substantially but not entirely (see discussion in 3.2. and 3.3.). This assessment should be updated regularly when new data, especially from long-term clinical studies or epidemiological investigations, become available. The impact of e-cigarettes and HTPs on public health is a different story that was not addressed during this

risk assessment. It needs to be better understood which factors and product characteristics lead to favorable (e.g., reduction of nicotine-use associated morbidity and mortality) and which to unfavorable public health outcomes (e.g., increasing nicotine-use initiation). For example, how much nicotine do ANDS need to deliver to keep smokers away from tobacco cigarettes? How much nicotine is too much, inducing addiction in naïve users? Which flavors are preferred by naïve users, especially by minors? Which flavors help former smokers to stay adherend to the alternative product? Some research on this topic is already available (e.g., as reviewed by Zare *et al.* [243]). However, caution should be taken when transferring population-based knowledge from one country to another. Thus, studies with suitable methods addressing user motivation and behavioral aspects are required for different regions and should also be performed for the population in Germany.

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6. List of publications

6.1. Publications included in this thesis

Mallock, N., Pieper, E., Hutzler, C., Henkler-Stephani, F., Luch, A. (2019). Heated Tobacco Products: A review of current knowledge and initial assessments. *Frontiers in public health*, *7:287*.

Mallock, N., Trieu, H. L., Macziol, M., Malke, S., Katz, A., Laux, P., Henkler-Stephani, F., Hahn, J., Hutzler, C., Luch, A. (2020). Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols. *Archives of Toxicology*, *94*, 1985-1994.

Mallock, N., Rabenstein, A., Laux, P., Rüther, T., Hutzler, C., Parr, M. K., Luch, A., (2021). Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans*-3'-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma. *Journal of Chromatography B, 1179*, 122736.

Mallock, N. & Rabenstein, A., Gernun, S., Laux, P., Hutzler, C., Karch, S., Koller, G., Henkler-Stephani, F., Parr, M. K., Pogarell, O., Luch, A., Rüther, T. (2021). Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after pod modification. *Scientific Reports, 11*, 12078.

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6.2. Other articles and book chapters

Pieper, E., **Mallock, N.**, Laux, P., Luch, A. (2020). E-Zigaretten/Tabakerhitzer – Risiken und Nutzen. In H. Stöver (Hrsg.), E-Zigaretten, Tabakerhitzer – was wir wissen müssen (S. 156-174). Frankfurt: Fachhochschulverlag.

Henkler-Stephani, F., **Mallock, N.**, Stephani, A., Pieper, E., Luch, A. (2019). Aktuelle Bewertung von E-Zigaretten und Tabakerhitzern. In H. Stöver (Hrsg.), *Potentiale der E-Zigarette für Rauchentwöhnung und Public Health* (S. 26-41). Frankfurt: Fachhochschulverlag.

Pieper, E., **Mallock, N.**, Henkler-Stephani, F., Luch, A. (2018). Tabakerhitzer als neues Produkt der Tabakindustrie: Gesundheitliche Risiken. *Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz*, *61*(11), 1422-1428.

6.2. Conferences

Talk – 18. Deutsche Konferenz für Tabakkontrolle, online 02.12.2020, “Suchtpotential von Juul: Die Nicotide-Studie”

Poster – 20th Annual Conference, Society For Research on Nicotine and Tobacco, online 17.09. - 18.09.2019, “JUUL e-cigarettes under European law – Lower nicotine content but higher vapor generation”

Poster – 19th Annual Conference, Society For Research on Nicotine and Tobacco, Oslo 12.09. – 14.09.2019 “Cold-receptor activation by tobacco products: L-Isopulegol is a potent alternative to menthol”

Poster – 4th German Pharm-Tox Summit, Stuttgart, 26.02. - 28.02.2019, “Potential promotion of attractiveness by cooling agents in tobacco: Investigations on enantioselective TRPM8 receptor activation”

Poster – Lebensmittelchemikertag, Berlin, 26.02.-01.03.2018, "Pyrolysis-gas chromatography/mass spectrometry for simulation of thermodegradation of a polylactide material used in tobacco products"

Talk – 18th Annual Conference, Society For Research on Nicotine and Tobacco, München, 06.09-08.09.2018, "Analysis of selected carcinogens in the emissions of "Heat not Burn" tobacco products"

Poster – 3rd German Pharm-Tox Summit, Göttingen, 26.02.-01.03.2018, "Levels of selected carcinogens in the emissions of “Heat not Burn” tobacco products"

Talk – 15. Deutsche Konferenz für Tabakkontrolle, Heidelberg, 06.12.-07.12.2017, "Gesundheitsgefährdung durch Tabakerhitzer: Was wir bisher wissen"

6.3. Meetings

Talk - JATC, Work Package 8 Meeting, Milan, 17.01. – 18.01.2019, “Testing of Heated Tobacco Products”

Talk - DIN Arbeitsausschuss "Tabak und Tabakerzeugnisse", Berlin, 04.12.2018, “Neuartige Tabakerzeugnisse”

Talk - Workshop on the Chemical Analysis of Traditional and Novel Tobacco Products, JRC Geel, Belgien, 11.10.-12.10.2018, "Testing of heated tobacco products"

Annex I: Supplementary Material

Trendy e-cigarettes enter Europe: chemical characterization of JUUL pods and its aerosols

Nadja Mallock, Hai Linh Trieu, Miriam Macziol, Sebastian Malke, Aaron Katz, Peter Laux, Frank Henkler-Stephani, Jürgen Hahn, Christoph Hutzler, and Andreas Luch

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Link: https://doi.org/10.1007/s00204-020-02716-3

HS-SPME method development for determination of benzoic acid in liquids and vapor

Selection of the separation columns and extraction fibers was based on the literature (Dong et al. 2006; Dong and Wang 2006). For any reliable quantification by HS-SPME-GC/MS, extraction needs to be performed under equilibrium conditions. Therefore, the incubation and extraction parameters were optimized. Different times and temperatures were tested in duplicate and the areas under the curves for benzoic acid and internal standards were compared. Some key optimization experiments are summarized in Figure 4.

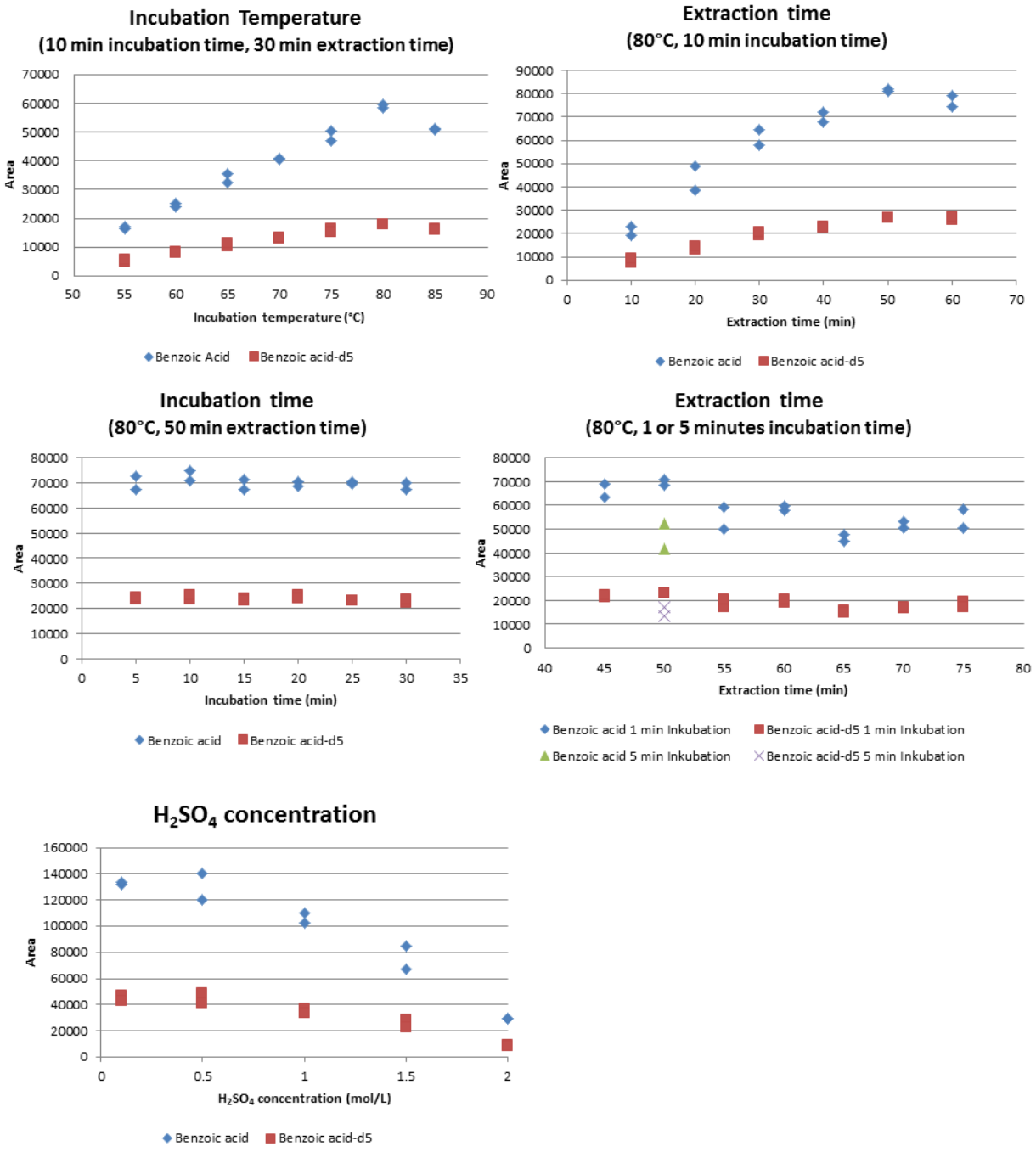

Figure 4. Optimization of Headspace Solid Phase Microextraction (HS-SPME) parameters

Aerosol generation - Comparison of two mouth pieces by two laboratories

The rectangular shape of JUUL made it difficult to directly connect the device to the smoking machine using rubber tubes. Our initial solution was the self-fabrication of a fitting mouth piece using a heat-shrinkable tubing (Figure 5b). When the manufacturer of the vaping machine offered a commercially available mouth piece (Figure 5a), we compared the vapor generation using both variants. In lab A, we analyzed 10 initial Rich Tobacco JUUL pods for each mouth piece. Total particulate matter (TPM) and liquid consumption were determined for a total of 20 fractions of 20 puffs each. Furthermore, to increase validity of our procedure, we analyzed 10 additional pods with the bought mouth piece in a different laboratory (lab B) for an inter-laboratory comparison.

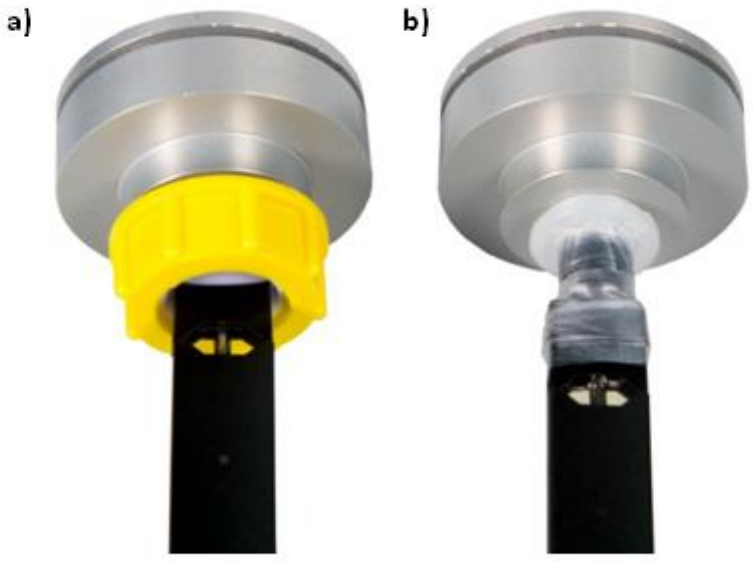

Figure 5. Two different mouth pieces for connection of the rectangular E-cigarette and the filter holder. Variant a) is commercially available, whereas variant b) is self-made with a heat-shrinkable tubing

The generated data for these two settings are displayed in Figure 6. For each collected fraction, the mean and standard deviation of the 10 analyzed pods were calculated. Four outcomes can be extracted: Firstly, the amount of consumed liquid correlates with the corresponding TPM collected. Secondly, the results from both laboratories are in good agreement. For the first 8 fractions (first 160 puffs), mean and standard deviation of TPM were 34 ± 6 mg and 32 ± 8 mg for lab A and lab B, respectively. Thirdly, in comparison of both mouth pieces in lab A, the commercially available mouth piece has led to more consistent results than the self-made variant. The self-made variant resulted in slightly higher but still acceptable results for the first 11 fractions. We assume that the connection between e-cigarette and mouth piece was slightly tighter for the self-made variant. As a

consequence, we hypothesize that the activator within the e-cigarette, which is only activated by a sufficient flow, might have started the power supply of the coil a little bit earlier for each puff. The resulting longer heating duration together with the slightly higher flow may be the reason for the small increase in vapor generation. However, for future analyses of e-cigarettes with unusual and challenging shapes, self-fabrication of mouth pieces using heat-shrinkable tubing is possible for the determination of TPM and nicotine. Fourthly, the standard deviations are high and increasing towards the end of the analysis. This can only be explained with a different performance of the pods with their included coils and wicks.

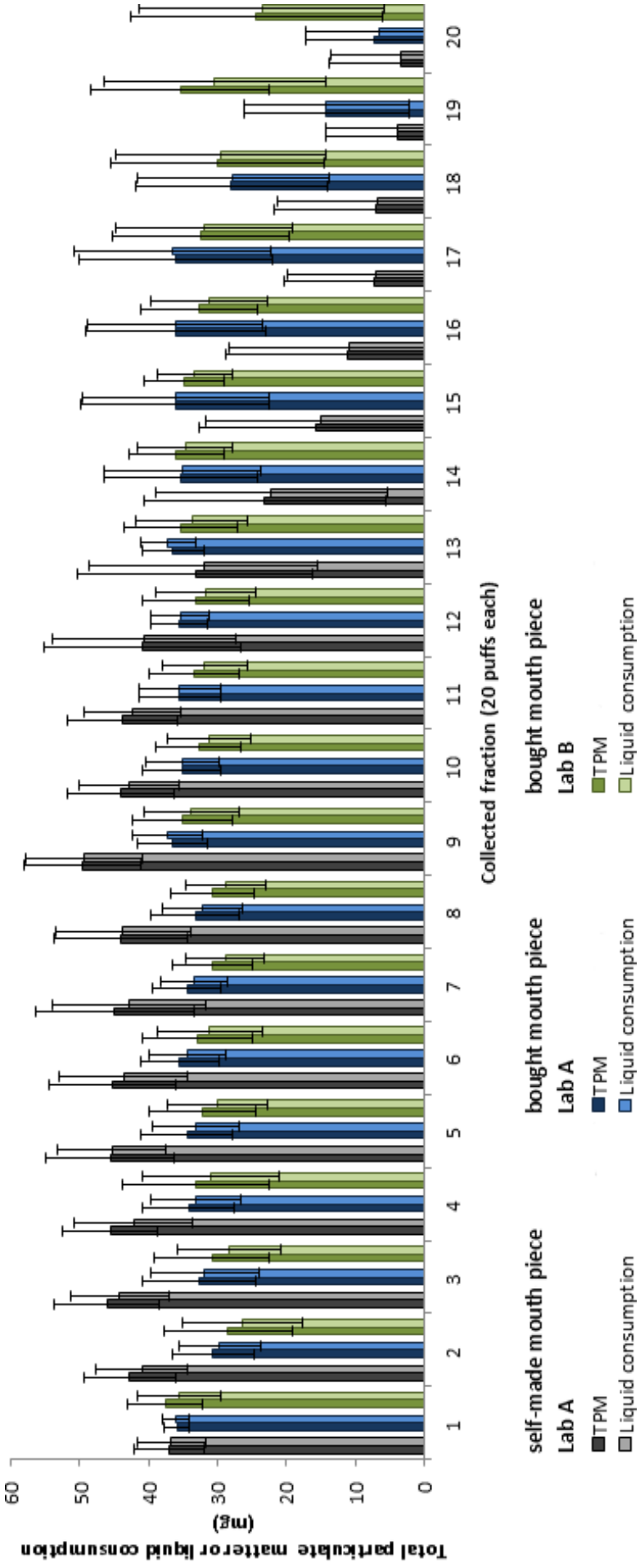

Figure 6. Mean and standard deviations of total particulate matter (TPM) and liquid consumption from 10 initial pods, analyzed in two different laboratories with two different mouth pieces

Aerosol generation by modified JUUL version

In lab A, TPM and liquid consumption of 6 modified Rich Tobacco JUUL pods with 9 and 18 mg/mL nicotine were analyzed using the commercially available mouth piece. TPM and liquid consumption are shown in Figure 7. With the modified pods, the deviation between fractions and the standard deviation between different pods decreased. This points to an improved consistency of vapor generation in the modified product.

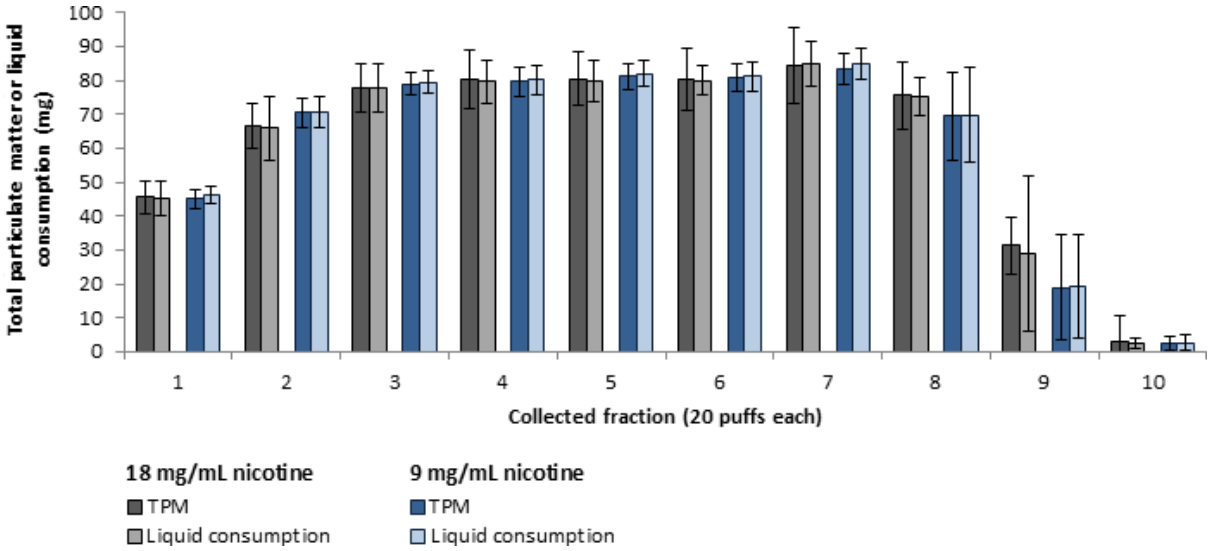

Figure 7. Mean and standard deviation of total particulate matter (TPM) and liquid consumption from 6 modified JUUL pods

Comparison of JUUL versions and mouth pieces regarding cumulative aerosol generation

Regarding the total liquid consumption over time in Figure 8, both JUUL variants and mouth pieces had a steady increase until about 600 mg were consumed. Afterwards, liquid was consumed much slower. The modified JUUL pods were nearly empty after 160 puffs, as already displayed in Figure 7. Pods of the initial version were nearly empty after roughly 300 or 340 puffs with the self-made and the bought mouth piece, respectively. As already visible in Figure 6, both mouth piece types show some differences in aerosol generation. However, the differences are small enough that the use of a self-made mouth piece is justified when a commercial option is not available. The modified JUUL version provides only half the number of puffs compared to the initial one. This could result in increasing in costs for the consumer.

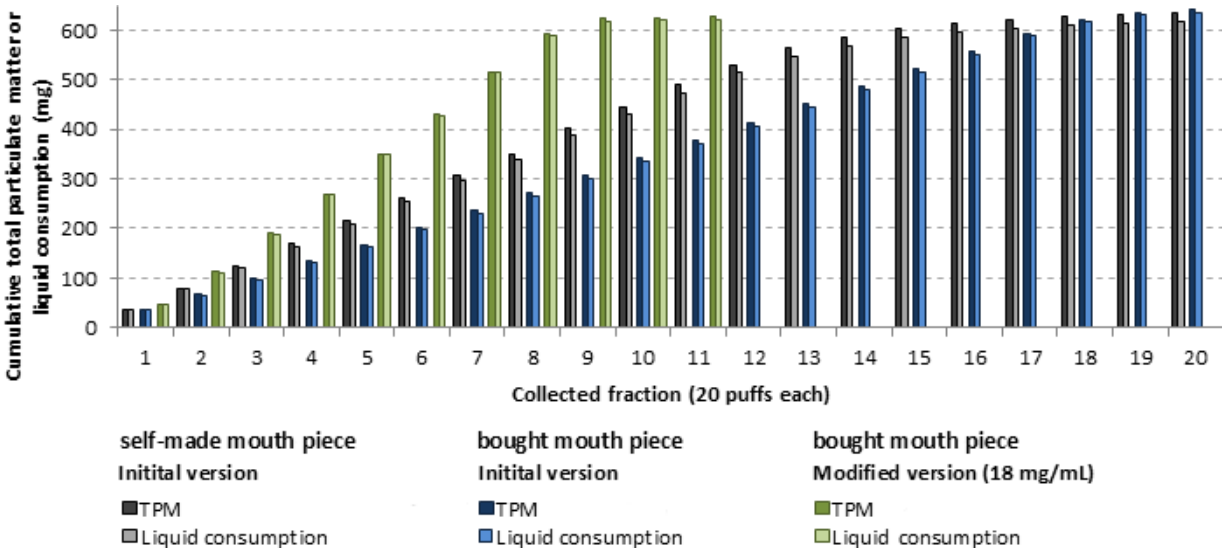

Figure 8. Cumulative total particulate matter (TPM) and liquid consumption from the mean of 6 modified JUUL pods and 10 initial JUUL pods, analyzed with different mouth pieces in lab A

Correlation between measured and calculated nicotine levels in the aerosol

The nicotine concentration in the aerosol was analyzed with GC/FID as described in the Methods section of the main manuscript. Additionally, the nicotine content can be calculated by multiplying the collected TPM with the nicotine concentration in the liquid (in mg/mg), as previously demonstrated by Talih et al. (Talih et al. 2017). To ensure quality, measured and calculated nicotine concentrations were compared using the data set from lab A for the initial Rich Tobacco pods with the commercially available mouth piece as an example. Only values above 10 mg TPM were included. As displayed in Figure 8, both ways to determine nicotine levels in the aerosol were in good agreement. Since a reliable quantification method was available in both laboratories, nicotine was measured with GC/FID. Nevertheless, the data imply that nicotine contents in the aerosol could be approximated only based on weighing of the e-cigarette, since the consumed liquid and the collected TPM have nearly the same mass as demonstrated in Supplementary Figures 6 and 7.

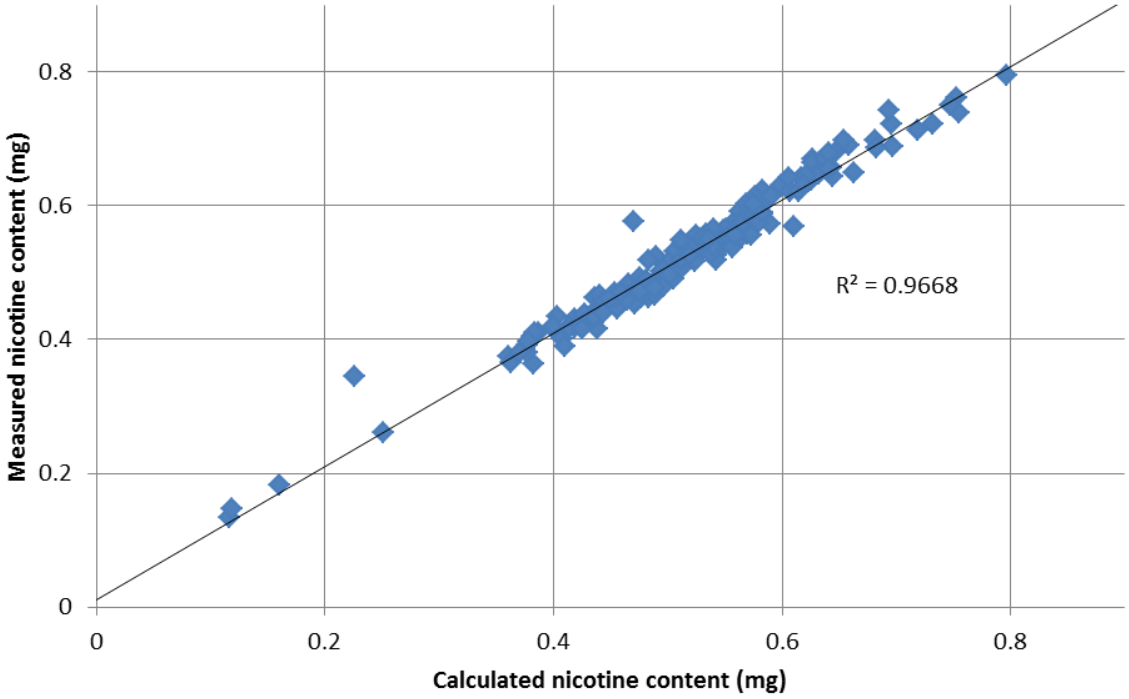

Figure 9. Correlation between measured and calculated nicotine contents in the aerosol

Analytical limits of carbonyl compound quantification

Limits of detection (LOD) and limits of quantification (LOQ) were estimated from the lowest standard (16.4 ng/puff) via the signal-to-noise ratio (S/N) and are presented in Table 2. S/N for LOD was 3 and for LOQ 5. Sample values between the lowest standard and LOQ were extrapolated; values between LOQ and LOD were set as the middle between LOQ and LOD.

Table 2. Limits of detection (LOD) and quantification (LOQ) for the analysis of carbonyl compounds calculated based on ng/puff

Analyte	Limit of detection (ng/puff)	Limit of quantification (ng/puff)
Formaldehyde	0.5	0.9
Acetaldehyde	1.9	3.2
Acetone	1.3	2.2
Acrolein	1.1	1.9

Continuity of carbonyl compound emission

In terms of carbonyl compound emissions, a “dry puff” effect has been shown in e-cigarettes by some groups (Farsalinos and Gillman 2017; Hutzler et al. 2014). This effect describes that at the end of consumption, when the liquid is too low to sufficiently supply wick and coil, temperature and consequently formation of carbonyl compounds can increase, resulting in harsher emissions (Farsalinos and Gillman 2017). To find out whether this occurs with JUUL in the machine smoking set up as well, the ratio between the amount of carbonyl compounds and the liquid consumption was evaluated. An increasing ratio in combination with a decreasing amount of consumed liquid indicates a spike in carbonyl formation. These ratios per collected fraction and the according liquid consumption values are displayed for one exemplary pod (modified JUUL version) in Supplementary Figure 10. The limit of quantification per 40 puffs of each analyte was divided by the corresponding liquid consumption and included in the figure. For all experiments, carbonyl formation was low during the first 160 puffs. Enhanced carbonyl formation was detected after a strong decrease of the amount of evaporated liquid per puff was recorded (below 20 mg). This was due to exhaustion of the liquid reservoir. Although "dry puff" conditions can theoretically occur, hardly any aerosol is generated at that stage. The small number of repeats and especially the quantification close to the analytical limits should be noted. Values for carbonyl emissions in ng/puff in Table 1 in the main text include only the first 160 puffs to represent normal test conditions without spikes due to “dry puffing”.

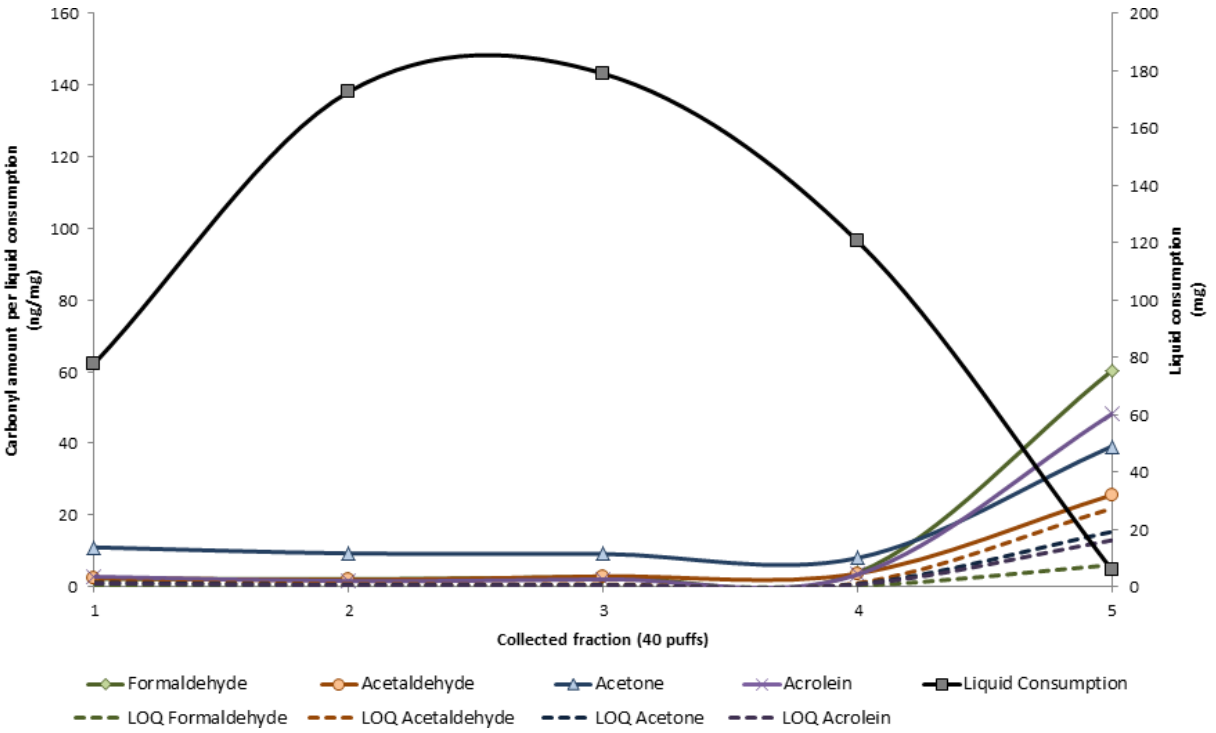

Figure 10. Carbonyl compound emissions by one modified JUUL pod in ng per mg consumed liquid (primary y-axis) and liquid consumption in mg (secondary y-axis) per collected fraction of 40 puffs each

FT-IR spectra of wick material

The differences in the used wick material are visible in the FT-IR spectra as seen in Figure 9. The American and the initial European JUUL versions deliver the same spectra, whereas the modified JUUL wick clearly consists of a different material. The spectrum of the modified JUUL wick in Figure 9a (Supplement) shows characteristic bands that are not present in the other two spectra: A wide band from 3600 to 3200 cm^{-1}, originating from hydroxyl groups, stretching vibrations from aliphatic (C-H)-bonds (3000 to 2800 cm^{-1}), and signals in the finger print region from 1500 to 1200 cm^{-1} (Hesse et al. 1991).

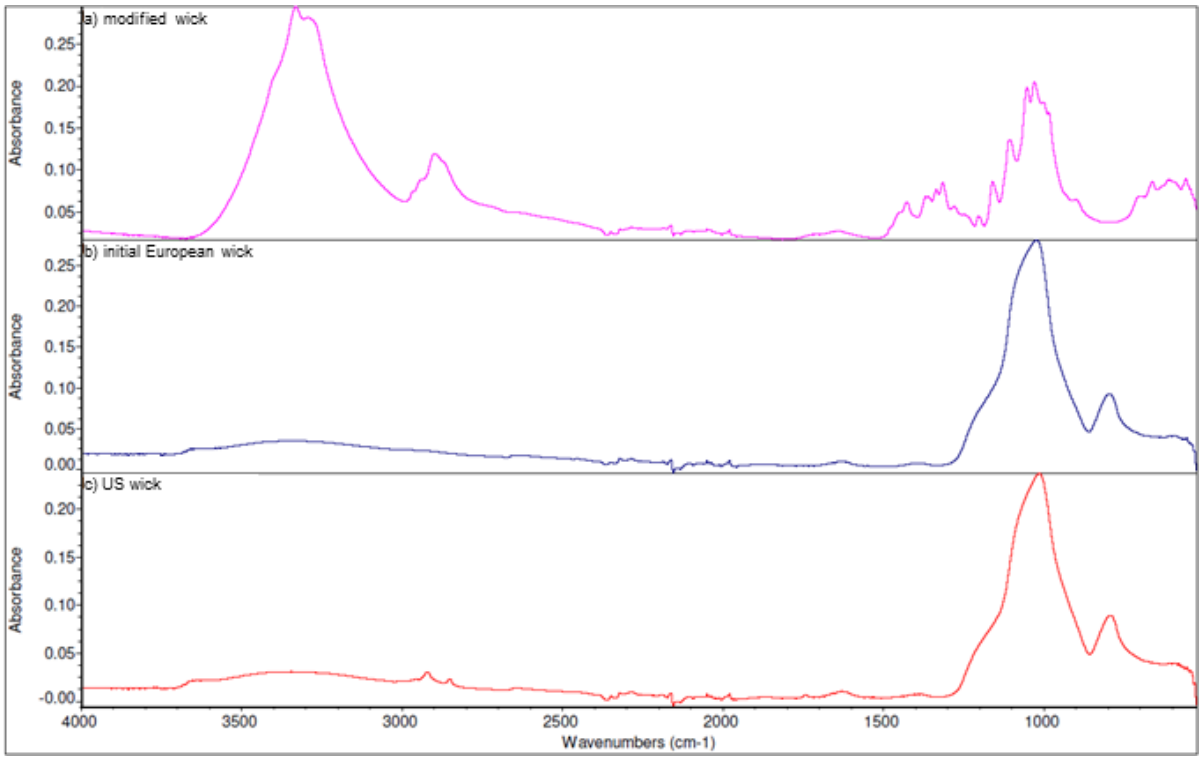

Figure 11. Attenuated total reflectance-Fourier-transform infrared (FT-IR) spectra of different wicks: a) modified JUUL, b) American-JUUL, and c) initial European JUUL

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Annex II: Supplementary Material

Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and trans-3'-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma

Nadja Mallock, Andrea Rabenstein, Peter Laux, Tobias Rüther, Christoph Hutzler, Maria Kristina Parr, and Andreas Luch

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Link: https://doi.org/10.1016/j.jchromb.2021.122736

Supplementary Material

Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans*-3'-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma

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Validation results: Selectivity

To assess selectivity of the method, six different blank matrices from different donors have been analyzed. Chromatograms of MRM transitions of analytes and internal standards are shown in Figure S.1 and Figure S.2, respectively. Nicotine (RT 3.22 min) and nicotine-d_3 (RT 3.20 min) transitions are displayed in pink (qualifier in lighter pink), hydroxycotinine (RT 4.38 min) and hydroxycotinine-d_3 (RT 4.35 min) are displayed in red (quantifier) and orange (qualifier), cotinine (RT 5.22 min) and cotinine-d_3 (RT 5.21 min) are displayed in blue (qualifier in darker blue). Except for some small signals in the MRM transition of cotinine qualifier, there are no interferences at the according retention times for both, analytes and internal standards. This interference could stem from small cotinine traces due to second hand nicotine exposure. However, there was no such interference in the quantifier transition. As discussed in the main text of the manuscript, the quantifier of cotinine has a lower response compared with the qualifier to enhance linearity of the working range. Furthermore, as seen in Figure 7 in the main text, the height of the cotinine qualifier signal at LLOQ (6 ng/mL) is about 1.2×10^5. The interferences in Figure S.1 are much lower than 20% of the response of the LLOQ as required by EMA guidelines. Selectivity of the method is sufficiently given.

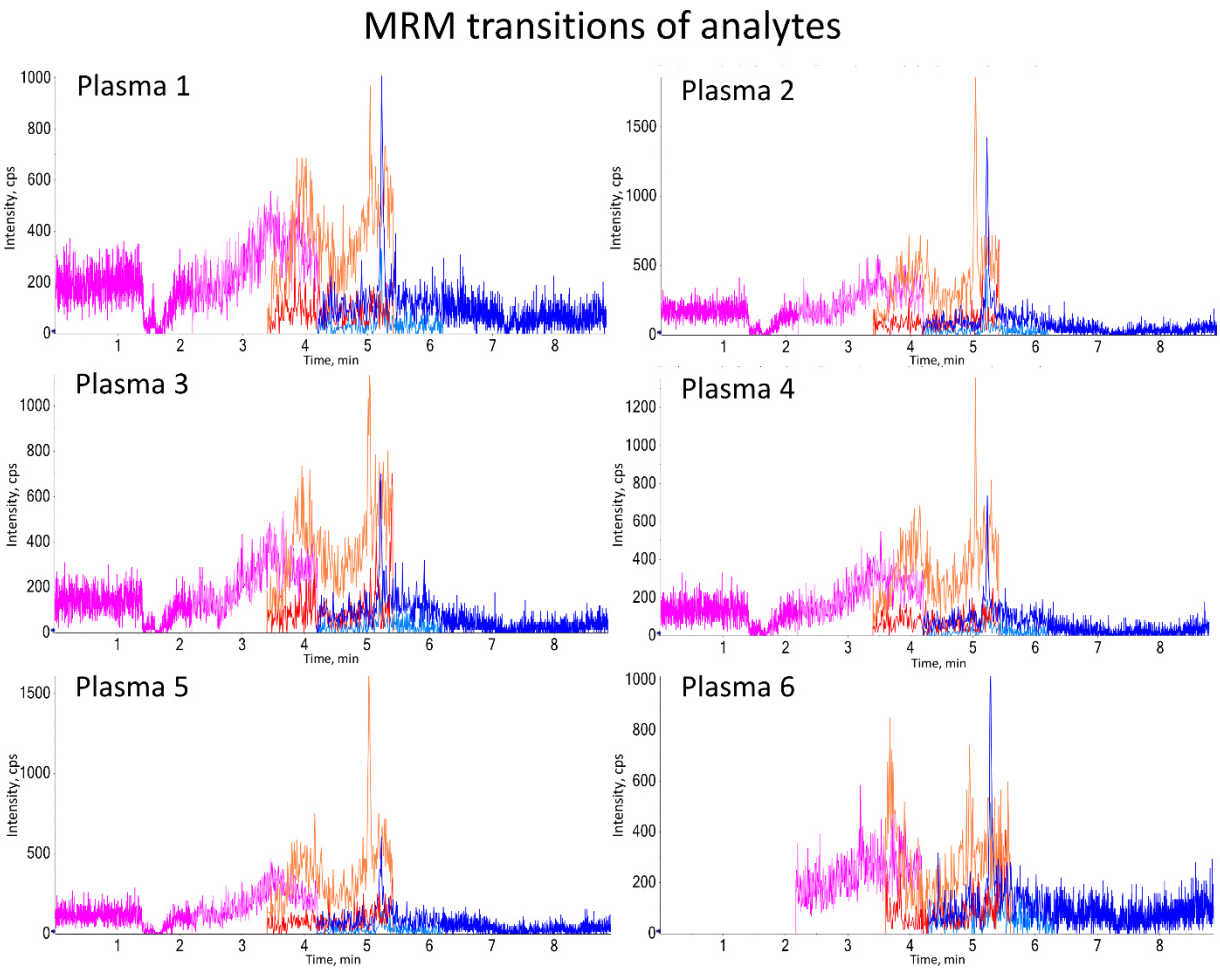

Figure S.1. MRM transitions of analytes in blank plasma derived from six different donors. Pink: Nicotine quantifier; lighter pink: nicotine qualifier; red: hydroxycotinine quantifier; orange: hydroxycotinine qualifier; light blue: cotinine quantifier; dark blue: cotinine qualifier

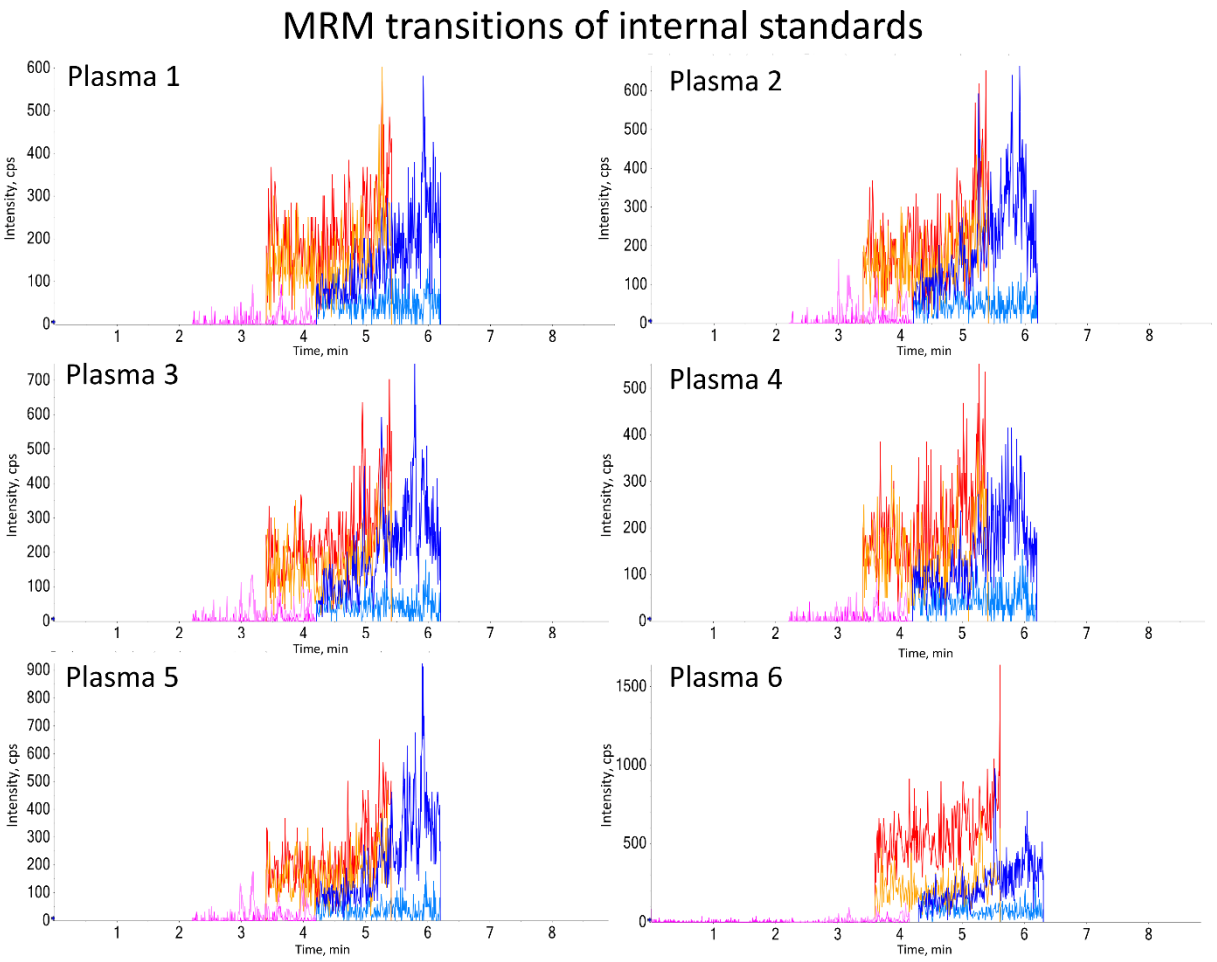

Figure S.2. MRM transitions of internal standards in blank plasma derived from six different donors. Pink: Nicotine-d_3 quantifier; lighter pink: nicotine-d_3 qualifier; red: hydroxycotinine-d_3 quantifier; orange: hydroxycotinine-d_3 qualifier; light blue: cotinine-d_3 quantifier; dark blue: cotinine-d_3 qualifier

Validation results: Linearity

Linear calibration curves (weighting 1/x) on day 1 of validation are displayed for all analytes in Figure S.3. Accuracies of both injections of calibration levels at the three validation days are summarized in Table S.1 including mean and standard deviation. More than 75% of standards were within 15% of nominal value, at LLOQ within 20%.

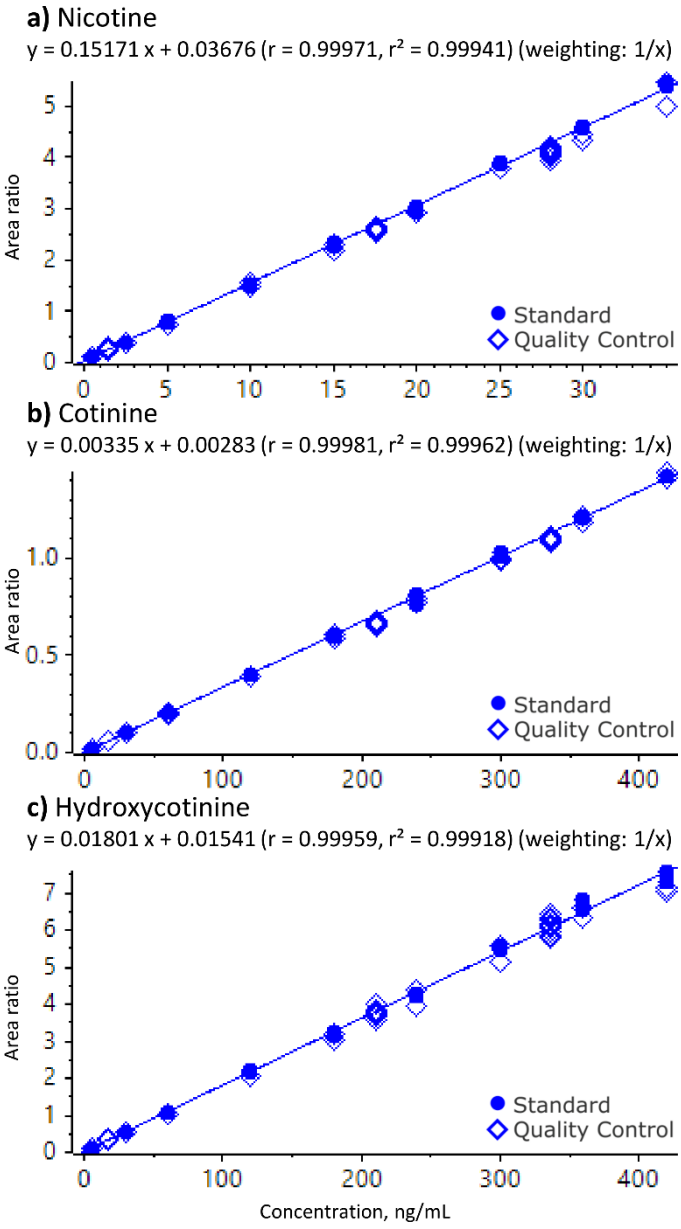

Figure S.3. Calibration curves of **a)** nicotine, **b)** cotinine, and **c)** hydroxycotinine, including quality control samples at 0.50, 1.50, 17.5, 28.0 ng/mL for nicotine and 6.00, 18.0, 210, 336 ng/mL for cotinine and hydroxycotinine (QC)

Table S.1. Accuracies of calibration standards at the three validation days including mean and standard deviations.

Standard (ng/mL)	Day 1 %	Day 1 %	Day 2 %	Day 2 %	Day 3 %	Day 3 %	Mean ± SD %
Nicotine							
K1 (0.50)	95.57	119.27	97.69	117.47	89.71	109.52	104.9 ± 12.3
K2 (2.50)	94.33	96.19	95.19	97.31	107.95	83.83	95.8 ± 7.7
K3 (5.00)	101.47	98.41	95.5	97.28	102.61	111.03	101.1 ± 5.6
K4 (10.0)	96.31	98.35	99.62	101.83	97.22	96.3	98.3 ± 2.2
K5 (15.0)	98.24	100.52	95.98	95.7	101.42	98.55	98.4 ± 2.3
K6 (20.0)	99.46	95.35	98.94	103.16	108.99	92.91	99.8 ± 5.7
K7 (25.0)	101.31	102.22	101.14	100.28	100.38	101.79	101.2 ± 0.8
K8 (30.0)	99.53	100.58	103.55	100.84	97.34	101.94	100.6 ± 2.1
K9 (35.0)	102.19	100.7	96.18	102.34	98.61	99.89	100.0 ± 2.3
Cotinine							
K1 (6.00)	95.95	108.59	97.53	99.94	96.19	98.68	99.5 ± 4.7
K2 (30.0)	97.96	99.92	97.81	103.97	101.48	101.82	100.5 ± 2.4
K3 (60.0)	101.14	99.59	102.04	100.13	100.85	98.89	100.4 ± 1.1
K4 (120)	98.81	99.7	99.22	97.98	101.65	97.95	99.2 ± 1.4
K5 (180)	97.96	100.96	98.69	99.38	101.22	102.92	100.2 ± 1.8
K6 (240)	100.36	94.78	102.99	103.02	102.74	101.86	101.0 ± 3.2
K7 (300)	101.89	100.23	100.04	98.29	100.03	98.43	99.8 ± 1.3
K8 (360)	100.56	99.56	98.47	101.43	93.04	97.25	98.4 ± 3.0
K9 (420)	101.06	100.96	100.85	98.23	103.02	102	101.0 ± 1.6
Hydroxycotinine							
K1 (6.00)	104.36	105.49	112.3	92.15	98.3	97.28	101.6 ± 7.2
K2 (30.0)	94.74	94.69	102.18	98.98	115.19	93.69	99.9 ± 8.2
K3 (60.0)	100.6	100.44	105.22	96.14	101.59	98.56	100.4 ± 3.0
K4 (120)	102.31	99.3	100.68	97.67	102.19	95.74	99.6 ± 2.6
K5 (180)	97.13	99.71	98.67	90.32	96.83	102.89	97.6 ± 4.2
K6 (240)	98.3	97.06	105.99	101.28	97.56	100.9	100.2 ± 3.3
K7 (300)	101	102.6	98.73	95.01	98.85	97.06	98.9 ± 2.7
K8 (360)	101.18	104.62	99.24	97.35	97.09	99.1	99.8 ± 2.8
K9 (420)	99.96	96.51	103.28	104.8	101.59	105.61	102.0 ± 3.4

Validation results: Stability

Stability of quality control samples (Low QC and High QC) under defined conditions was determined. For acceptance, the mean concentrations had to be 85 – 115% of the nominal value. For assessment of benchtop stability, QCs were left at room temperature or on ice and were prepared for analysis at 0, 30, 60, 90, 120, 180, 240, and 300 min in triplicate. As presented in Table S.2., stability was given for all three analytes in both concentrations at room temperature and on ice for at least 5 h.

Table S.2. Benchtop stability at room temperature (RT) or on ice of quality control samples prior to preparation as mean recoveries out of three determinations.

Minute		0	30	60	90	120	180	240	300
QC (ng/mL)	Condition								
Nicotine									
Low QC (1.5)	RT	95.7%	98.5%	88.8%	85.0%	95.6%	90.5%	88.8%	86.2%
Low QC (1.5)	Ice	95.7%	94.8%	89.8%	100.9%	106.3%	90.6%	94.6%	91.6%
High QC (28)	RT	91.9%	91.4%	93.7%	96.5%	95.2%	92.9%	92.4%	92.7%
High QC (28)	Ice	91.9%	95.5%	97.0%	92.5%	101.3%	92.9%	93.8%	93.8%
Cotinine									
Low QC (18)	RT	98.0%	95.6%	97.4%	98.9%	94.6%	93.5%	96.8%	96.7%
Low QC (18)	Ice	98.0%	93.4%	95.3%	98.1%	95.3%	104.4%	93.9%	94.7%
High QC (336)	RT	99.4%	98.8%	99.1%	97.1%	97.5%	99.3%	98.3%	98.1%
High QC (336)	Ice	99.4%	96.8%	96.7%	99.1%	95.7%	97.8%	98.7%	100.2%
Hydroxycotinine									
Low QC (18)	RT	110.3%	110.2%	100.2%	102.2%	111.4%	113.1%	107.0%	111.4%
Low QC (18)	Ice	110.3%	104.4%	95.8%	109.8%	102.3%	104.3%	105.0%	103.6%
High QC (336)	RT	110.1%	108.1%	105.6%	107.0%	109.7%	111.9%	112.4%	110.8%
High QC (336)	Ice	110.9%	110.9%	108.5%	106.3%	106.4%	106.0%	107.2%	109.6%

Quality control samples were analyzed directly after their production on day 1 (freeze and thaw cycle 0) and kept at -80°C for at least 12 h before they were thawed and analyzed against a freshly produced matrix calibration (freeze and thaw cycle 1). The procedure was repeated with the same quality control samples for two further freeze and thaw cycles with at least 12 h at -80°C and analysis against freshly produced matrix calibrations. As displayed in Table S.3., stability was given for all three analytes in both concentrations for at least three freeze and thaw cycles.

Table S.3. Freeze and thaw stability of quality control samples prior to preparation as mean recoveries out of three determinations.

Freeze and thaw cycle	0	1	2	3
QC (ng/mL)				
Nicotine				
Low QC (1.5)	98.6%	94.0%	104.2%	97.6%
High QC (28)	95.1%	96.1%	99.6%	94.6%
Cotinine				
Low QC (18)	100.9%	97.3%	103.7%	98.0%
High QC (336)	96.7%	95.6%	101.7%	98.6%
Hydroxycotinine				
Low QC (18)	104.4%	109.4%	99.5%	108.2%
High QC (336)	100.7%	106.4%	109.8%	106.2%

Stability at autosampler conditions (15 °C) was tested with the matrix calibration samples for 24 hours. As presented in Table S.4, samples were stable in the autosampler for at least 24 h.

Table S.4. Stability of prepared matrix samples under autosampler conditions at 15°C over 24 h as mean recoveries

QC (ng/mL)	Beginning of sequence	After 24 h
Nicotine		
Low QC (1.5)	103.2%	112.7%
High QC (28)	100.0%	101.0%
Cotinine		
Low QC (18)	99.9%	101.9%
High QC (336)	99.0%	99.2%
Hydroxycotinine		
Low QC (18)	107.2%	111.8%
High QC (336)	106.1%	113.8%

Validation results: Matrix factor

Internal standard (IS)-normalized matrix factors were determined according to EMA Guideline on bioanalytical method validation with venous plasma from six different donors (matrix A – F) [1]. Calculation is described below:

$$\text{Matrix factor} = \frac{\text{Peak area of analyte in spiked matrix}}{\text{Peak area of analyte in matrix-free sample}}$$

$$\text{IS-normalized matrix factor} = \frac{\text{Matrix factor of analyte}}{\text{Matrix factor of IS}}$$

According to guidelines, CV of IS-normalized matrix factors from six different lots of matrix should be ≤ 15 %. As displayed in Table S.5, this acceptance criterion for matrix effects has been met.

Table S.5. IS-normalized matrix factors (MF) of six different human plasma matrices (mean of 3 runs) for low QC and high QC for nicotine, cotinine, and hydroxycotinine.

	Nicotine		**Cotinine**		**Hydroxycotinine**	
Matrix	Low QC (1.5 ng/mL)	High QC (28 ng/mL)	Low QC (18 ng/mL)	High QC (336 ng/mL)	Low QC (18 ng/mL)	High QC (336 ng/mL)
A	1.09	1.13	1.37	1.37	1.48	1.29
B	1.06	1.15	1.45	1.41	1.44	1.32
C	1.11	1.14	1.47	1.38	1.48	1.31
D	1.20	1.13	1.57	1.36	1.58	1.25
E	1.07	1.16	1.45	1.35	1.54	1.31
F	1.07	1.12	1.39	1.38	1.43	1.26
Mean	1.10	1.14	1.45	1.38	1.49	1.29
SD	0.05	0.02	0.07	0.02	0.06	0.03
CV	4.6%	1.3%	4.9%	1.5%	3.8%	2.3%

Validation results: Intra-laboratory repeatability

Additional to validation following guidelines on bioanalytical method validation [1], other parameters not required by EMA such as intra-laboratory repeatability were tested with matrix quality control samples at other concentrations. Results are presented in Table S.6. The differences between operators did not exceed 20%. Mean accuracies ranged from 93.1% to 118.1%. The precisions of sample preparation and the instrument were below 10% within one day and between two days. The method was repeatable and reproducible within the laboratory.

Table S.6. Overview of results of additional validation experiments for intra-laboratory repeatability

Concentration ng/mL	Mean accuracy (operator 1)		Precision of sample preparation		Instrument precision		Mean accuracy (operator 2) (%)
	Day 1 (%)	Day 2 (%)	Intra-day (%)	Inter-day (%)	Intra-day (%)	Inter-day (%)	
Nicotine							
0.75	101.6	110.6	5.5	6.5	7.5	7.1	118.1
12.5	111.1	109.8	1.5	2.1	1.4	1.9	109.2
22.5	110.4	107.4	3.1	2.6	1.4	1.6	107.1
32.5	103.3	102.1	1.6	1.4	1.3	1.6	103.5
Cotinine							
9.00	99.2	100.8	1.5	1.5	2.4	2.5	100.6
150	107.7	106.4	1.9	2.0	1.9	2.0	105.4
270	103.7	102.5	0.7	1.1	1.6	2.1	102.4
390	94.5	94.4	2.5	1.9	2.3	2.1	93.1
Hydroxycotinine							
9.00	100.9	103.2	3.3	3.7	3.6	3.7	104.2
150	108.4	108.1	1.1	1.3	2.3	3.1	105.3
270	102.8	103.7	2.0	2.5	2.0	2.9	103.3
390	96.1	94.0	2.5	2.5	2.9	2.3	94.0

Product ion spectra

Product ion mass spectra of reference substance solutions (50 - 100 ng/mL in methanol) are displayed in Figure S.4. Spectra were derived during compound optimization with a syringe pump and a flow of 15 µL/min using standard parameters: Ion spray voltage, 5500 V; ion source temperature, off; curtain gas, nitrogen with 10 psi; ion source gas 1, nitrogen with 10 psi; ion source gas 2, nitrogen with 0 psi; declustering potential, 61 V; entrance potential, 10 V; collision energy, 52 V; collision exit potential, 10 V.

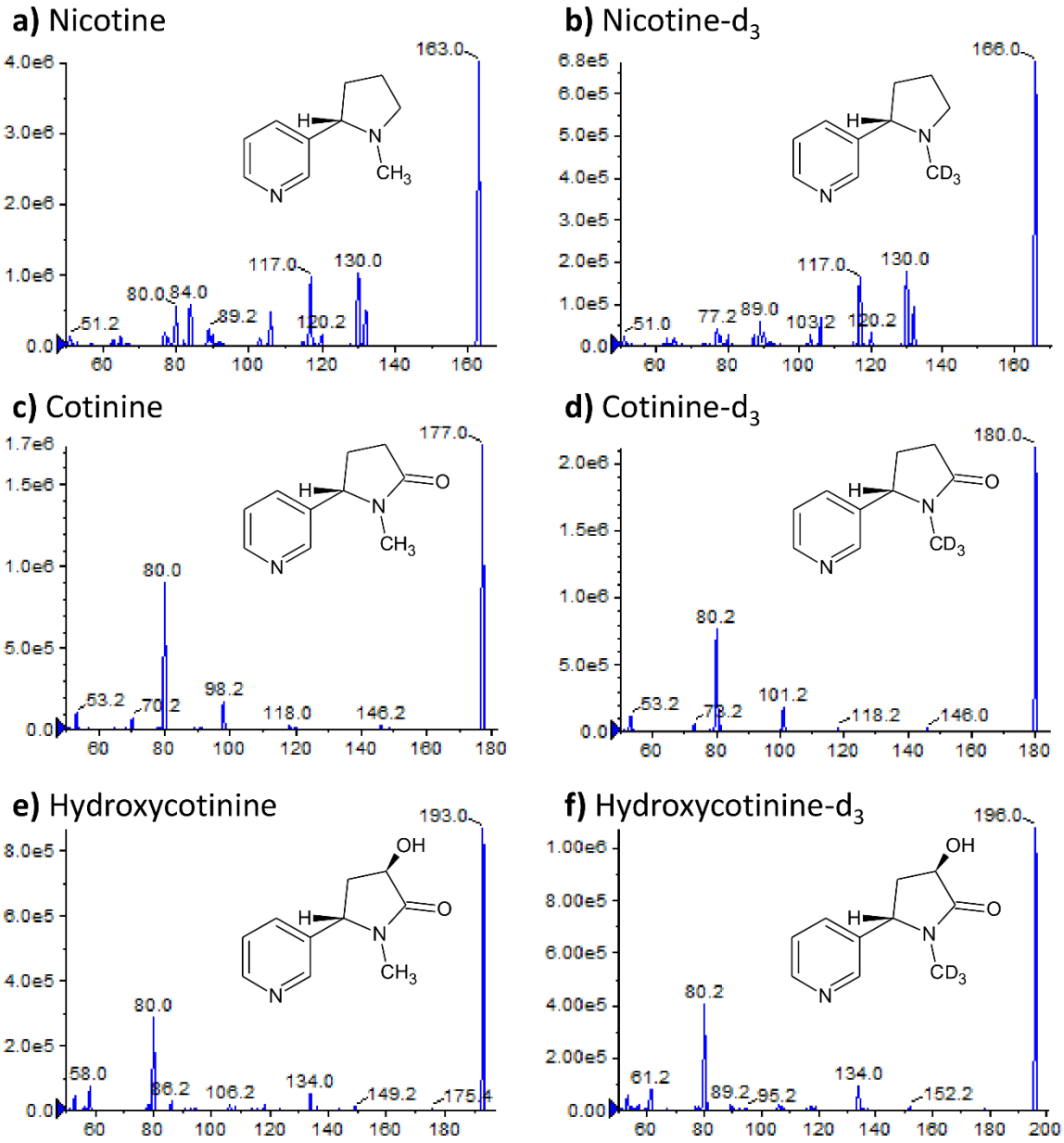

Figure S.4. Product ion sprectra with chemical structures of **a)** nicotine (parent ion: 163.0 Da), **b)** nicotine-d_3 (parent ion: 166.0 Da), **c)** cotinine (parent ion: 177.0 Da), **d)** cotinine-d_3 (parent ion: 180.0 Da), **e)** hydroxycotinine (parent ion: 193.0 Da), and **f)** hydroxycotinine-d_3 (parent ion: 196.0 Da).

Reference

[1] European Medicines Agency, Guideline on bioanalytical method validation, 2015.

Annex III: Supplementary Material

Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after pod modification

Nadja Mallock*, Andrea Rabenstein*, Solveig Gernun, Peter Laux, Christoph Hutzler, Susanne Karch, Gabriele Koller, Frank Henkler-Stephani, Maria Kristina Parr, Oliver Pogarell, Andreas Luch, and Tobias Rüther

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Online Supplementary Material

Nicotine delivery and relief of craving after consumption of European JUUL e-cigarettes prior and after product modification

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Supplementary information on methodology

Questionnaire on Smoking Urges (QSU-G)

The Questionnaire on Smoking Urges (QSU) consists of 32 items which are rated on a scale from 1-7 (totally disagree to totally agree). The German version of the QSU (QSU-G) was published in 2001 after a validation study in Germany and has since become an established clinical instrument to evaluate smoking behavior[1]. Participants completed the QSU-G before and after the study visits to determine their craving for smoking. The QSU-G assesses two factor-specific dimensions of subjective craving for smoking on a seven-level rating scale. Factor 1 describes the intention to smoke and anticipation of positive effects from smoking (positive reinforcement, positive reinforcement). Factor 2 indicates the craving for smoking and anticipation of relief from negative effects of nicotine withdrawal (negative reinforcement, negative reinforcement).

For data analysis, test items were grouped into both factors. While the items 4,5,6,9,11,17,21,25,27,28 and 32 represent factor 1 (positive reinforcement), the items 2,3,7,13,18,19,24,29,30 and 31 were assigned to factor 2 (negative reinforcement). For the evaluation of the questionnaire the Items 4,6,8,10,11,16,17,21,22,26,27,28 and 32 needed to be recoded. For example, a score of 7 in item 4 had to be recoded into a score of 1.

Analysis of nicotine, cotinine, and hydroxycotinine plasma concentrations

Multiple reaction monitoring (MRM) mode was used for mass selective detection in positive ionization mode with a 120s detection window and 1s cycle time. Two characteristic fragmentation reactions were used as transitions per analyte. Parameters for ESI source and scheduled MRM are given in Supplementary Tables 1 and 2.

Supplementary Table 1. Ion source parameters for nicotine, cotinine, and hydroxycotinine quantitation method.

Ion spray voltage	3800 V
Ion source temperature	650 °C
Curtain gas	N_2, 10 psi
Ion source gas 1	N_2, 80 psi
Ion source gas 2	N_2, 85 psi
Declustering potential	47 V
Entrance potential	7 V

Supplementary Table 2. MRM parameters for nicotine, cotinine, and hydroxycotinine quantitation method.

Analyte	Retention time	Quantifier Q1 (Da) → Q3 (Da)	CE (V)	CXP (V)	Qualifier Q1 (Da) → Q3 (Da)	CE (V)	CXP (V)
Nicotine	3.2 min	163.2 → 130.0	29	6	163.2 → 132.1	21	24
Cotinine	5.2 min	177.2 → 98.0	40	18	177.2 → 80.0	25	14
Hydroxycotinine	4.4 min	193.1 → 80.0	43	14	193.1 → 134.1	27	24
Nicotine-d_3	3.2 min	166.3 → 132.0	23	6	166.3 → 130.0	45	6
Cotinine-d_3	5.2 min	180.2 → 80.0	35	14	180.2 → 101.0	31	18
Hydroxycotinine-d_3	4.4 min	196.2 → 80.0	41	14	196.2 → 134.1	27	24

CE: Collision energy

CXP: Collision exit potential

Supplementary information on results

Plasma nicotine levels at different time points

Supplementary Table 3. Results of plasma analysis for cigarette smokers. Excluded or missed values are highlighted in grey.

Cigarette smoker	NMR	Plasma nicotine (ng/mL)									AUC_{0-30} ng/mL*min
		t0	1 min	2 min	4 min	6 min	8 min	10 min	12 min	30 min	
01	1.40	0.2	0.3	1.1	3.5	6.4	**7.4**	6.4	7.4	7.8	187.3
02	0.39	0.8	7.3	21.9	28.9	**37.4**	20.5	16.6	17.6	9.4	483.7
03	0.46	0.5	1.2	3.8	7.5	**9.8**	N/A	N/A	9.9	11.4	267.5
04	0.67	0.5	0.7	1.1	4.4	5.3	7.6	7.2	**8.1**	7.3	183.7
05	0.45	2.4	2.3	7.5	13.1	**19.3**	N/A	N/A	11.7	8.8	266.4
06	0.63	0.3	0.4	0.8	5.4	7.0	8.2	**10.3**	N/A	8.8	226.2
07	0.48	6.0	6.0	5.8	7.8	9.8	11.4	**12.4**	11.5	9.8	125.0
08	nq	0.0*	0.0*	0.0*	0.0*	0.1*	0.1*	0.1*	0.1*	0.0*	N/A
09	0.58	0.1	0.4	2.3	5.6	**8.6**	7.9	6.5	N/A	6.0	178.4
10	0.29	5.5	10.3	14.8	21.8	25.5	24.2	**26.3**	25.9	14.5	454.4
11	0.62	0.2	0.6	7.8	21.5	**26.6**	21.3	9.7	N/A	4.5	297.4
12	0.61	0.1	0.2	0.7	3.4	6.8	10.0	**10.8**	9.2	5.4	201.0
13	0.25	0.3	0.9	2.4	6.9	**7.8**	7.7	6.4	6.4	N/A	N/A
14	0.16	0.6	1.0	3.1	10.9	17.1	**17.1**	N/A	N/A	11.3	374.5
15	0.28	0.0	0.5	7.1	18.3	**29.1**	18.0	15.3	14.4	3.9	351.5

NMR: Nicotine metabolic ratio (hydroxycotinin/cotinin concentration at t_0)

AUC_{0-30}: Area under the curve t_0-t_{30min} (after subtraction of $C_{(t0)}$)

N/A: not assessed (no blood sampling or no AUC_{0-30} calculation possible)

nq: not quantified (at least one metabolite concentration not quantifiable)

*: Not included in calculation of mean values

Supplementary Table 4. Results of plasma analysis for users of new/modified JUUL e-cigarettes. Excluded or missed values are highlighted in grey.

Users new JUUL	NMR	Used liquid (mg)	Nicotine dose (mg)	Plasma nicotine (ng/mL) t0	1 min	2 min	4 min	6 min	8 min	10 min	12 min	30 min	AUC_{0-30} ng/mL*min
01	0.27	N/A	N/A	0.7	0.9	2.6	4.6	5.0	**5.0**	N/A	3.8	2.4	82.3
02	0.42	32.6	0.50	0.1	0.2	1.4	4.4	5.2	**6.5**	4.5	N/A	1.7	96.6
03	0.23	28.7	0.44	1.2	2.1	3.3	**5.8**	5.7	4.3	3.8	3.3	2.4	67.4
04	0.36	41.3	0.63	4.4	4.7	8.1	12.3	**14.2**	12.4	10.6	9.7	7.0	146.6
05	0.37	40.5	0.62	2.5	8.1	11.5	12.8	**15.4**	11.9	N/A	N/A	8.0	239.3
06‡	0.56	37.8	0.58	0.0	1.0	6.1	**11.5**	9.4	7.0	4.9	4.7	4.3	161.5
06•	*0.55*	*28.9*	*0.44*	*0.9*	*1.1*	*3.9*	*N/A*	*N/A*	*N/A*	*4.2*	*4.1*	*3.4*	*N/A*
07	nq	36.8	0.56	0.0	0.1	5.4	6.0	**7.0**	5.3	N/A	N/A	1.9	118.8
08‡	0.51	33.7	0.51	1.0	2.9	4.4	4.9	**5.4**	4.5	4.0	3.9	3.0	82.7
08•	*0.93*	*32.9*	*0.50*	*0.3*	*1.6*	*4.5*	*4.2*	*N/A*	*N/A*	*2.3*	*3.2*	*1.6*	*N/A*
09	0.39	32.2	0.49	0.4	1.5	2.7	5.0	**6.9**	4.6	N/A	N/A	N/A	N/A
10	nq	23.8	0.36	0.1	0.3	1.6	2.6	**2.9**	N/A	1.8	1.6	1.0	44.7
11	0.27	20.1	0.31	0.4	0.9	N/A	3.0	N/A	N/A	N/A	N/A	N/A	N/A
12	0.39	30.6	0.47	4.5	16.4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
13‡	0.56	17.4	0.27	0.9	3.3	5.3	**7.8**	8.2	5.7	N/A	3.7	N/A	N/A
13•	*0.42*	*21.5*	*0.33*	*4.3*	*2.2*	*9.7*	*N/A*	*N/A*	*N/A*	*N/A*	*N/A*	*3.8*	*N/A*
14‡	0.16	46.0	0.70	0.0	1.2	6.6	**11.2**	9.9	7.0	5.5	4.6	3.6	156.6
14•	*0.42*	*36.0*	*0.55*	*0.0*	*N/A*	*N/A*	*N/A*	*N/A*	*2.9*	*3.6*	*2.9*	*1.8*	*N/A*
15	0.46	25.2	0.38	0.5	1.4	**4.4**	3.6	3.3	N/A	N/A	N/A	2.3	68.7

NMR: Nicotine metabolic ratio (hydroxycotinin/cotinin concentration at t_0)

AUC_{0-30}: Area under the curve t_0-t_{30min} (after subtraction of $C_{(t0)}$)

N/A: not assessed (no blood sampling or no AUC_{0-30} calculation possible)

nq: not quantified (at least one metabolite concentration not quantifiable)

‡: Second recruitment due to missing t_{max} sampling

•: Omitted (first) measurement due to missing t_{max} sampling

Supplementary Table 5. Results of plasma analysis for users of old/initial JUUL e-cigarettes. Excluded or missed values are highlighted in grey.

Users old JUUL	**NMR**	**Used liquid (mg)**	**Nicotine dose (mg)**	**Plasma nicotine (ng/mL)**									**AUC_{0-30} ng/mL*min**
				t0	1 min	2 min	4 min	6 min	8 min	10 min	12 min	30 min	
01	0.59	44.5	0.68	0.4	4.3	**10.2**	8.9	8.9	5.9	4.8	4.4	2.8	133.4
02	0.44	36.2	0.55	1.5	4.1	8.6	**11.7**	10.9	8.9	N/A	5.9	3.5	140.9
03	0.98	42.8	0.65	0.2	0.4	1.0	**3.0**	2.7	2.9	2.5	2.8	1.7	60.8
04	0.27	32.6	0.50	5.2	6.9	8.8	11.0	**11.9**	10.8	9.8	10.4	8.6	135.1
05	0.21	28.0	0.43	0.4	4.2	**9.6**	9.5	7.9	5.6	5.1	N/A	3.0	138.5
06	0.25	29.1	0.44	1.7	2.1	3.7	**5.7**	4.9	5.2	**N/A**	5.4	3.9	87.6
07	0.27	32.2	0.49	0.0	1.6	6.5	**9.1**	8.3	5.9	5.4	4.4	2.4	134.8
08	0.43	13.9	0.21	1.1	1.4	3.2	**4.1**	3.8	4.0	3.5	3.5	N/A	N/A
09	0.47	38.1	0.58	10.2	17.6	**24.7**	22.5	20.4	17.6	16.1	15.8	12.6	178.4
10	0.51	29.8	0.45	0.0	9.0	**9.9**	5.9	5.3	4.7	4.1	N/A	2.0	121.0
11	0.26	8.9	0.14	1.2	2.0	3.0	**4.6**	4.3	3.4	N/A	N/A	2.1	51.7

NMR: Nicotine metabolic ratio (hydroxycotinin/cotinin concentration at $_{t0}$)

AUC_{0-30}: Area under the curve t_0-t_{30min} (after subtraction of $C_{(t0)}$)

N/A: not assessed (no blood sampling, metabolite concentration not quantifiable or no AUC_{0-30} calculation possible)

FTND and QSU-G scores for individual participants

Supplementary Table 6. FTND and QSU-G scored for tobacco cigarette smokers. Excluded or missed values are highlighted in grey.

Cigarette smoker	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
FTND	1	2	4	0	4	1	0	0*	0	7	0	0	1	0	1
QSU-G Factor 1 before	4.27	6.27	5.82	4.00	6.00	4.00	3.64	N/A	4.64	6.55	4.45	2.64	4.73	4.00	3.91
QSU-G Factor 1 after	4.27	5.18	6.09	2.18	3.82	4.09	3.82	N/A	5.09	4.55	2.64	2.45	3.82	3.09	2.18
QSU-G Factor 2 before	2.40	3.40	2.30	1.40	3.90	1.70	2.20	N/A	2.50	1.60	3.50	2.10	2.50	1.00	2.60
QSU-G Factor 2 after	2.60	2.30	2.90	1.10	2.60	1.80	2.10	N/A	3.10	1.40	2.20	2.00	2.00	1.00	1.90

*: Not included in calculation of mean values

N/A: no QSU-G score calculation due to prior exclusion

Supplementary Table 7. FTND and QSU-G scored for users of new/modified JUUL. Excluded or missed values are highlighted in grey.

New JUUL user	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15
FTND	2	0	8	5	5	3	0	2	1	2	7	8	2	3	8
QSU-G Factor 1 before	3.36	3.64	6.27	4.00	6.00	6.45	3.64	2.73	1.09	5.18	6.73	N/A	5.36	3.00	7.00
QSU-G Factor 1 after	4.64	5.09	6.91	1.73	4.55	5.55	2.91	3.09	3.91	4.00	5.55	N/A	3.73	3.27	7.00

QSU-G Factor 2 before	1.30	1.40	4.80	1.50	2.10	4.80	1.60	1.70	1.00	2.40	2.40	N/A	2.80	1.30	4.10
QSU-G Factor2 after	1.10	1.30	4.90	1.00	2.50	2.50	1.60	2.20	1.00	1.60	2.80	N/A	1.20	1.80	5.50

N/A: no score calculation due to incomplete participation in questionnaire

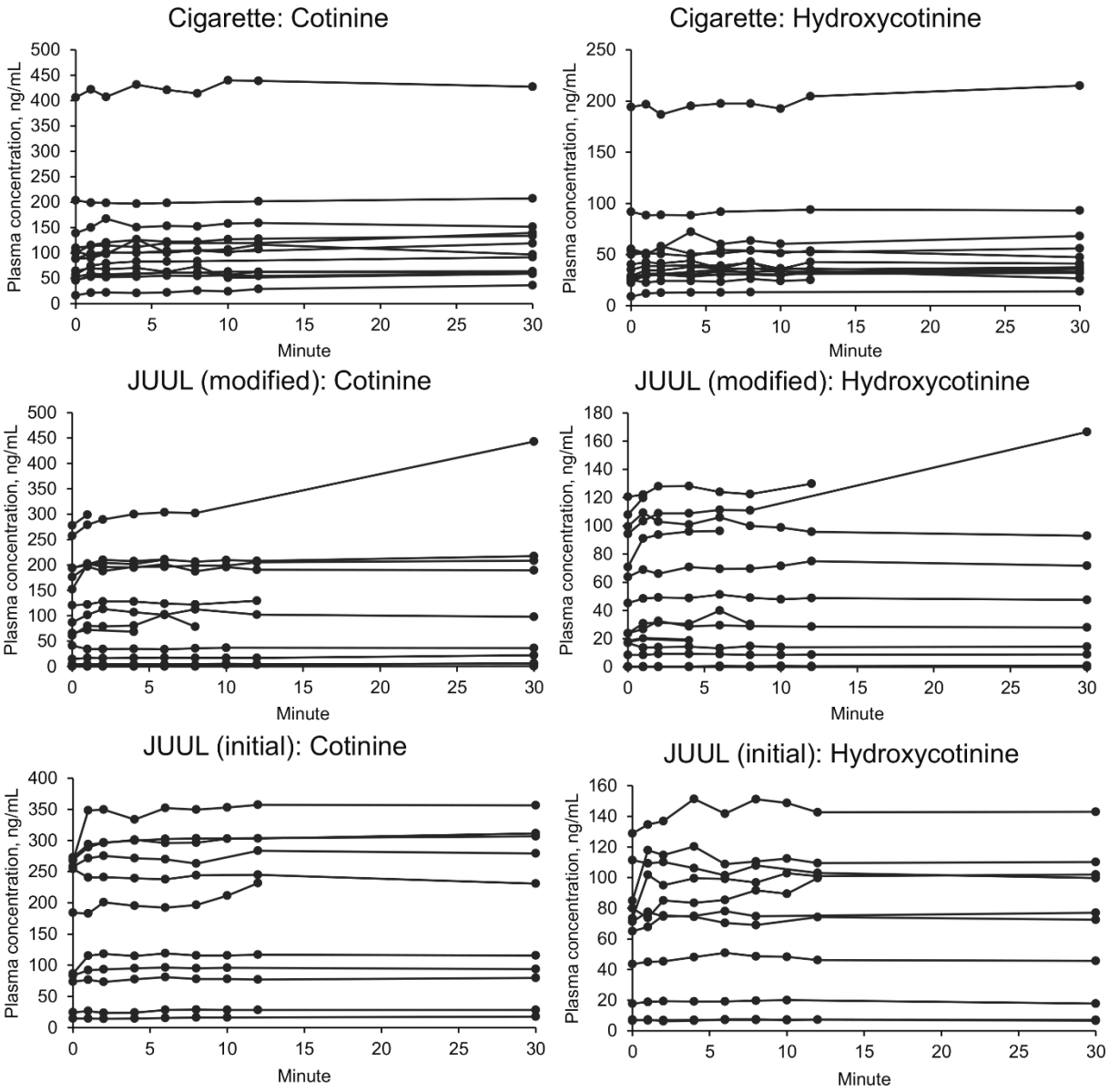
Supplementary Table 8. FTND and QSU-G scored for users of old/initial JUUL. Excluded or missed values are highlighted in grey.

Old JUUL user	01	02	03	04	05	06	07	08	09	10	11
FTND	0	5	2	5	2	8	0	5	8	3	N/A
QSU-G Factor 1 before	4.09	4.91	2.27	3.55	N/A	6.82	3.45	4.82	6.27	3.27	N/A
QSU-G Factor 1 after	5.18	4.36	3.45	2.82	N/A	6.82	2.82	5.27	6.64	3.64	N/A
QSU-G Factor 2 before	1.20	3.10	1.80	1.10	N/A	5.20	1.80	1.80	2.30	1.90	N/A
QSU-G Factor2 after	1.80	2.80	2.40	1.00	N/A	4.50	1.50	1.80	2.90	2.30	N/A

N/A: no score calculation due to incomplete participation in questionnaire

Cotinine and hydroxycotinine plasma concentration-time curves

Ratios of the plasma concentrations of metabolites hydroxycotinine at t_0 were calculated as a surrogate for nicotine metabolism. However, metabolites were determined at the other time points as well. Plasma concentration-time curves per group and analyte are presented in Supplementary Figure 1 for sake of completeness.



Supplementary Figure 1. Individual plasma concentration-time curves for metabolites cotinine and hydroxycotinine derived from the three study groups.

References

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Annex IV: Supplementary Material

Levels of selected analytes in the emissions of “heat not burn” tobacco products that are relevant to assess human health risks

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Supplementary Material

1. Materials and Methods

1.1. Tobacco heating devices and tobacco sticks

Tobacco heating devices (Smith et al., 2016) and two variants of the corresponding tobacco sticks containing different tobacco blends were purchased at local stores in Berlin, Germany. The heating devices were cleaned according to the recommendations of the manufacturer after every 18 runs using the provided cleaning sticks. The tobacco sticks were conditioned at 22 ± 1°C, 60 ± 2% rH for at least 48 hours (ISO 3402, 1999). Four heating devices with different usage histories were used as displayed in Supplementary Table 1.

Supplementary Table 1. Overview on the different tobacco heating devices and their history of usage prior to this study.

Device	History of usage
Device I	Unused
Device II	Used by a consumer for approximately one month and tested in a smoking machine with about 80 tobacco sticks
Device III	Applied in a smoking machine (about 60 tobacco sticks)
Device IV	Applied in a smoking machine (about 60 tobacco sticks)

1.2. Chemicals and standard substances

All chemicals and solvents were of analytical grade or higher. Acetonitrile and orthophosphoric acid (85%) were purchased from Merck KGaA (Darmstadt, Germany) and methanol from Merck Millipore (Billerica, MA, USA). 2,4-Dinitrophenylhydrazine (moistened with 33% water) was obtained from PanReac AppliChem (Darmstadt, Germany), hydranal solution from Honeywell Fluka (Hydranal-Composite 5, Morris Plains, NJ, USA). 2-Propanol containing the internal standards ethanol (2 g/L) and n-heptadecane (0.3 g/L) was bought from LGC Standards (Teddington, UK), and tris(hydroxymethyl)aminomethane from Sigma-Aldrich (St. Louis, MO, USA). Carbon dioxide for the generation of dry ice was obtained from Air Liquide (Paris, France). Analytical standards for benzene (99.96%), benzene-d_6 (99.96%), 1,3-butadiene (99.5%), isoprene (>99.5%), styrene (99.9%), toluene (99.9%) and the 2,4-dinitrophenylhydrazone (DNPH) derivatives of the carbonyl compounds acetaldehyde (99.9%), acrolein (99.5%), crotonaldehyde (99.9%) and formaldehyde (99.9%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (*S*)-nicotine salicylate (99.8%) was purchased from LGC Standards (Teddington, UK).

1.3. Generation of mainstream smoke

The mainstream smoke of tobacco sticks was generated using an LM4E smoking machine (Borgwaldt, Hamburg, Germany) with a PM1 piston pump unit (Borgwaldt, Hamburg, Germany) applying the Health Canada Intense smoking regimen (Health Canada, 2000) in order to maximize the number of drawn puffs. Tobacco heating devices were activated by button pushing for 3 s followed by a 20 s heating interval before the first puff was taken. Puff volumes of 55 mL were drawn within 2 s (puff duration) at a frequency of 30 s. Due to the device heating time of

6 min, a maximum of 12 puffs were taken. After the heating was switched off automatically, an additional clearing puff was performed. Differing from the HCI protocol, filter tips of the tobacco sticks were not covered with tape.

1.4. Determination of total particulate matter (TPM), nicotine, water and nicotine-free dry particulate matter (NFDPM) per tobacco stick and TPM and nicotine per three puffs

Mainstream smoke of three tobacco sticks was collected on a Cambridge glass-fiber filter pad (Ø 44 mm, Borgwaldt Köber Solutions, Hamburg, Germany). After gravimetric determination of TPM (CP 225D-0CE, Sartorius, Göttingen, Germany) the glass-fiber filter pad was extracted with 50 mL of isopropanol containing the internal standard n-heptadecane (0.3 g/L) on a shaker (SM-30 Control, Edmund Bühler , Hechingen, Germany) for at least 30 min at 60 rpm. The water content was analyzed with Karl-Fischer titration (841 Titrando, 803 TiStand, Metrohm, Filderstadt, Germany) using 5 mL of the extract and two to three titrations per sample. Nicotine was quantified by gas chromatography applying flame ionization detection at 300.0°C (7890A, Agilent Technologies, Santa Clara, CA, USA; 30 mL/min H_2 flow, 99.999%; 400 mL/min air flow; 15 mL/min make up flow, N_2, 99.999%; Air Liquide, Paris, France) on an HP-5 column (30 m x 0.530 mm, 2.65 µm film, Agilent Technologies, Santa Clara, CA, USA). 1.0 µL of extract was injected in splitless mode at the injection temperature of 250.0°C. The flow rate of the carrier gas helium (99.999%, Air Liquide, Paris, France) was 5.50 mL/min. The oven was programmed with the following temperatures: hold at 120.0°C for 5 min; a linear increase to 230.0°C for 6 min; hold at 230.0°C for 5.5 min. Nicotine and water contents were subtracted from the TPM to calculate nicotine-free dry particulate matter (NFDPM). For each combination of the four devices and two tobacco stick variants, six replicates were analyzed.

To examine the continuity of nicotine release into the mainstream smoke four intervals comprising three individual puffs (12 puffs per stick in total) were analyzed. The mainstream smoke for each interval was collected on separate glass-fiber filter pads per interval. TPM was determined gravimetrically. The mainstream smoke of the respective interval of three tobacco sticks was combined on the same filter for subsequent nicotine analysis: The glass-fiber filter pads were extracted with 20 mL of isopropanol containing 0.3 g/L n-heptadecane. Nicotine was quantified as mentioned above.

1.5. Determination of carbonyl compounds

The mainstream smoke of three tobacco sticks was not filtered by a Cambridge glass-fiber filter pad, but directly carried through two impingers containing 35 mL 2,4-dinitrophenylhydrazine solution in a row. After 30 min derivatization time, 8 mL of the sample solution was stabilized with 2 mL tris(hydroxymethyl)aminomethane solution (16 mg/mL). For devices I and II, five replicates were generated for each tobacco stick variant while four replicates were generated for devices III and IV.

DNPH derivatives of formaldehyde, acetaldehyde, crotonaldehyde and acrolein were quantified by liquid chromatography (1100 series: binary pump G1312 A, degasser G1312 A, column oven G1312 A, autosampler G1312 A, Agilent Technologies, Santa Clara, CA, USA) coupled to diode array detection at 360 nm (DAD G1312 A, Agilent Technologies, Santa Clara, CA, USA) and equipped with an RP-Amid column (Ascentis, 150 x 2 mm, 3 µm, Supelco, Bellefonte, PA, USA). The separation was carried out at 20°C and a gradient elution with water (eluent A) and acetonitrile (eluent B) using the following gradient: 0 - 10 min, a linear gradient from 40% to 50% B; hold for 4 min; 14 - 26 min, a linear gradient to 80% B; hold for 2 min. The flow rate was 300 µL/min. Injection

volume was 20 µL. All analytes were qualified by comparing the retention time to standards. Acetaldehyde-DNPH, crotonaldehyde-DNPH and acrolein-DNPH were also qualified by their UV spectra. The presence of all analytes in the sample was confirmed by LC-MS/MS (binary pump G1312 A, degasser G1379 B, column oven G1316 B, Agilent Technologies, Santa Clara, CA, USA; autosampler PAL HTS, PAL Systems, CTC Analytics AG, Zwingen, Switzerland; mass spectrometer API 4000, Sciex, Framingham, MA, United States) in negative mode with two transitions per analyte: m/z = 209 to 151 and 120 for formaldehyde, m/z = 223 to 151 and 76 for acetaldehyde, m/z = 235 to 181 and 158 for acrolein and m/z = 249 to 181 and 163 for crotonaldehyde.

1.6. Determination of volatiles and semi-volatiles

Mainstream smoke of nine tobacco sticks filtered by a Cambridge glass-fiber filter pad was carried through two impingers containing 10 mL of methanol in a cold trap. The temperature of the cold trap was held below –70°C with dry ice and isopropanol. Afterwards both sample solutions were spiked with 400 µg benzene-d_6 each and combined. Samples were stored in closed microvials at –20°C. For devices I and II three replicates were generated per tobacco stick variant. All samples were injected and analyzed in duplicate.

Quantification of 1,3-butadiene, benzene, isoprene, styrene and toluene was performed using an Agilent HP 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with an injector 7683 (split/splitless, Agilent Technologies, Santa Clara, CA, USA) and an HP-Plot Q column (30 m x 0.32 mm, 20 µm film, Agilent Technologies, Santa Clara, CA, USA) and a mass spectrometer Agilent MSD 5973. A helium gas flow of 7.0 mL/min was used with the following temperature program: hold 150°C for 5 min; linear increase to 170°C at 5°C/min; linear increase to 220°C at 20°C/min and hold for 1.5 min; linear increase with 5°C/min up to 260°C and final hold for 4 min. 1 µL of each sample was automatically injected in split mode with a split ratio of 5:1 and an inlet temperature of 250°C. The temperatures of the electron ionization ion source and the quadrupole were 230 and 180°C, respectively. Acquisition was performed in selective ion monitoring (SIM) mode, with the following five groups: start at 3.0 min the m/z ratios 27, 39, 50, 51, 53, 54 with dwell times of 40 ms; after 6.0 min the m/z ratios 26, 39, 51, 52, 53, 67, 68 with dwell times of 32 ms; after 10.0 min with the m/z ratios 50, 56, 77, 78, 82, 84 with dwell times of 40 ms; after 14.0 min the m/z ratios 39, 65, 91, 92 with 67 ms dwell time; after 18.0 min the m/z ratios 77, 78, 103, 104 and 67 ms dwell time.

2. Results in detail

2.1. Carbonyl compounds

The yields of the carbonyl compounds formaldehyde, acetaldehyde, acrolein and crotonaldehyde were determined (Supplementary Figure 1). Mainstream smoke was generated with four different heating devices and two tobacco stick variants. During our experimental procedure, device IV stopped working without noticeable reason. Thus the data set with this device could not be completed. Due to insufficient baseline separation, levels of crotonaldehyde were only assessed semi-quantitatively and are not presented in Figure 1. The threshold was set at 3.0 µg/stick and all samples resulted in concentrations below the threshold.

2.2. Volatile and semi-volatile compounds

The yields of the volatile and semi-volatile compounds benzene, 1,3-butadiene, isoprene, styrene and toluene are illustrated in Supplementary Figure 2. Each bar represents one device and one tobacco stick variant. Only the newest (I) and the oldest (II) device have been used to address this issue.

2.3. Inconsistent release of nicotine in the initial puffs

Each tobacco stick was smoked with 12 puffs. These 12 puffs were divided into four intervals of three puffs each: puffs 1 to 3 (interval 1, I1); puffs 4 to 6 (interval 2, I2); puffs 7 to 9 (interval 3, I3) and puffs 10 to 12 (interval 4, I4). Therefore, I1 represented the beginning of the smoking procedure whereas I4 resembled the end. As shown in Supplementary Figure 3, the TPM yields for both variants and for all four devices are the highest in the beginning of the smoking procedure (I1 with 14 mg and I2 with 14.8 mg as the mean of both variants) and then decrease during the second half of the smoking process with a minimum yield at the end (I4 with 8.8 mg for both variants). The dispersion (n=4) is also higher for I1 and I2 than for I3 and I4, indicating that the variability of the TPM yield is lower in the second half of the smoking procedure. However, the nicotine yield shows a minimum in the beginning (I1) and a maximum in the middle of the smoking process (I2 and I3). At the end it decreases slightly (I4). Nicotine levels were initially lower than 50% of the levels found in the middle of the smoking procedure and therefore represent only 10-12% of the total nicotine yield.

3. References

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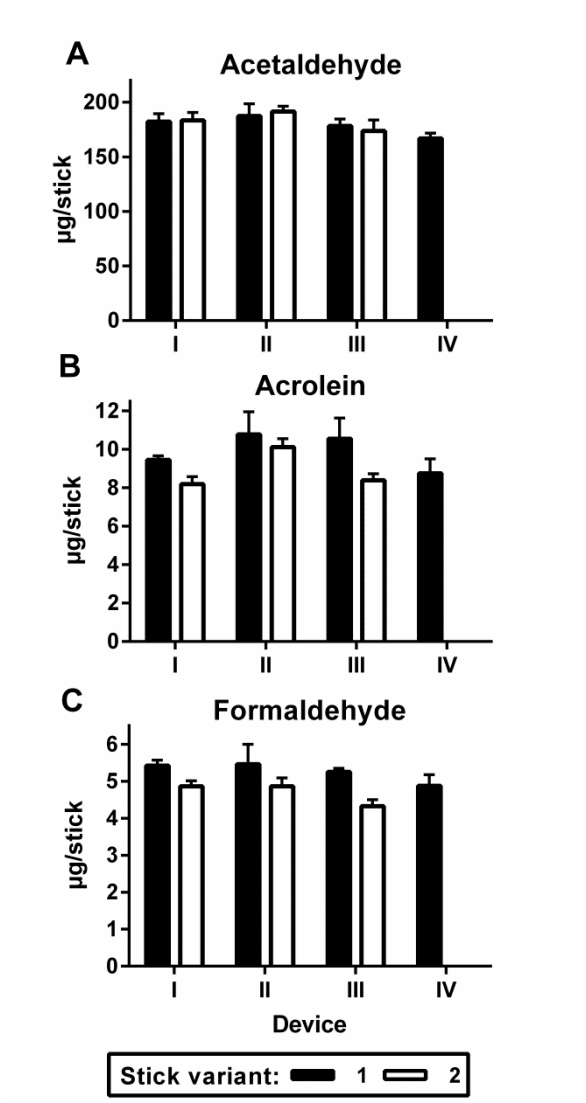
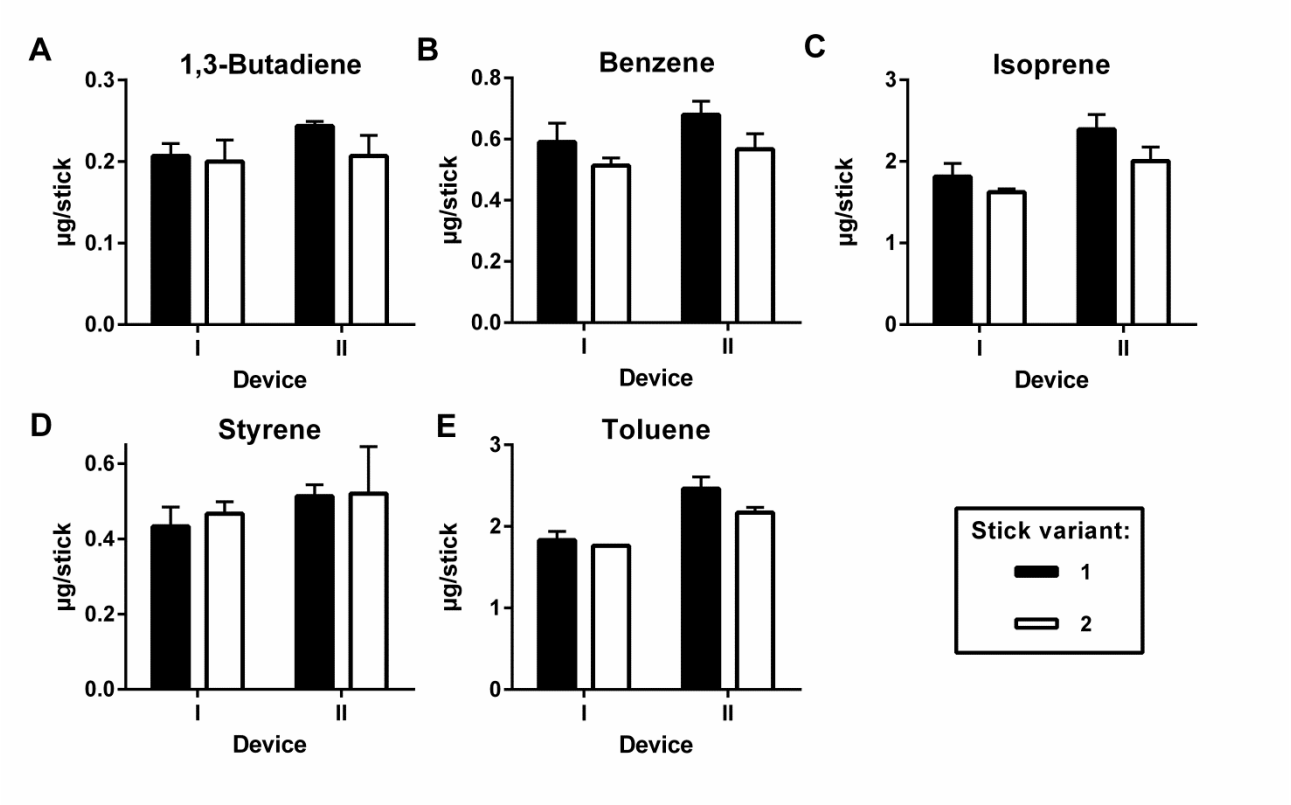
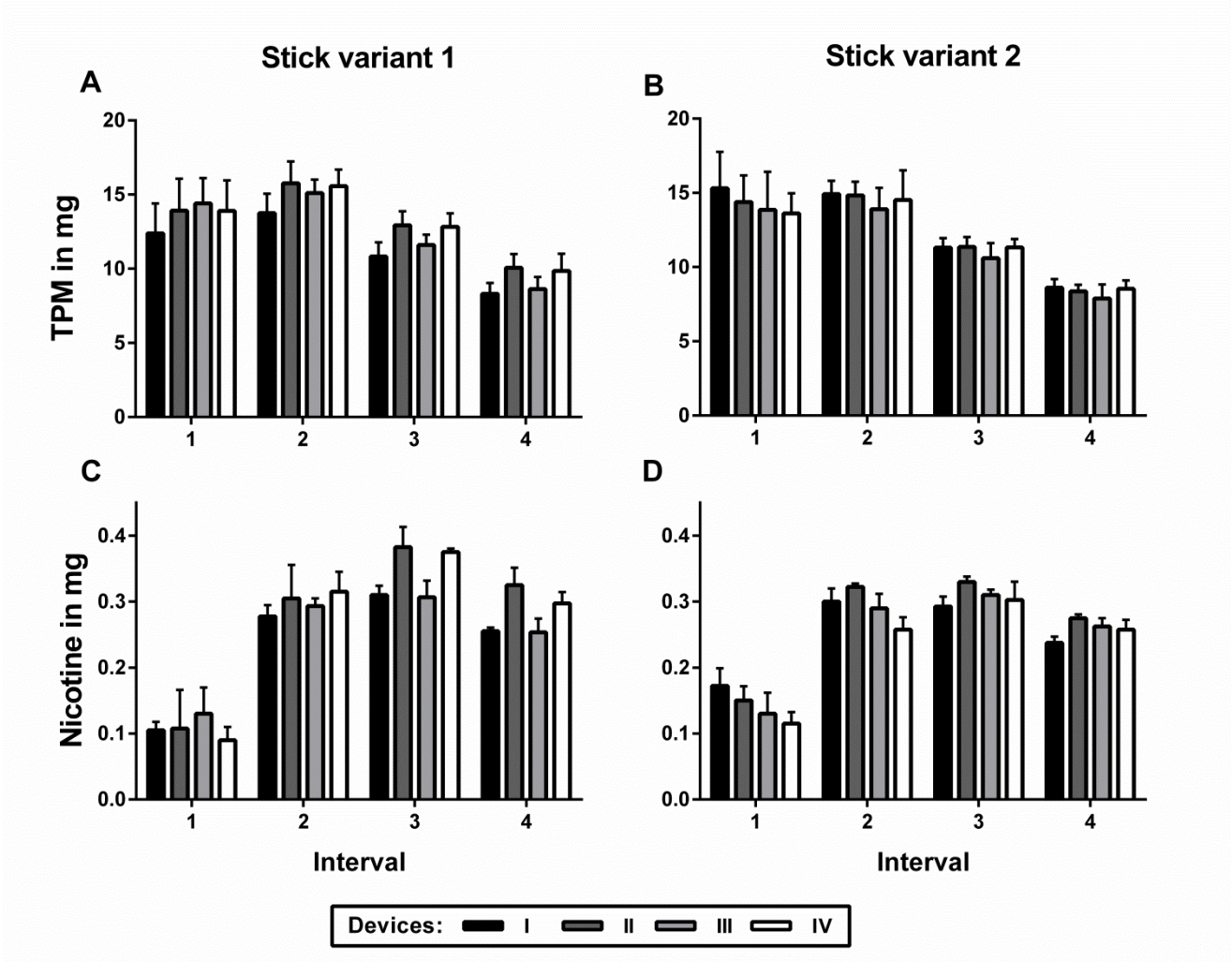


Supplementary Figure 1. Yields of the carbonyl compounds acetaldehyde (A), acrolein (B) and formaldehyde (C) generated with two different stick variants and the devices I, II, III and IV. Determination of crotonaldehyde was semi-quantitative and is therefore not displayed. There were 5 measurements (repeats) for devices I and II, and 4 for devices III and IV. Device IV stopped working before stick variant 2 could be assessed.



Supplementary Figure 2. Levels of the volatile compounds 1,3-butadiene (A), benzene (B), isoprene (C), styrene (D), and toluene (E) generated with two different stick variants. A new device (I) and the device with the longest usage history in this study (II) were used. There were 3 measurements (repeats) with double determination each.



Supplementary Figure 3. For machine smoking of one tobacco stick 12 puffs were conducted. These 12 puffs were divided into four intervals of three puffs each: interval 1 with puffs 1 – 3, interval 2 with puffs 4 – 6, interval 3 with puffs 7 – 9, and interval 4 with puffs 10 – 12. Stick variant 1 (A, C) and variant 2 (B, D) were smoked with the devices I, II, III, IV. For TPM (A, B) determination the number of repeats was 12, for nicotine (C, D) 4 with the exception of device III with variant 1 (C). Here only 3 repeats were performed.