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Biomarker-based dosimetry for e-cigarette users using stable-isotope labeled precursors and MS/MS analysis

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Anne Heidi Landmesser
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Biomarker
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(Methyl)-thiazolidine carboxylic acid

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A. Landmesser, M. Scherer, G.Scherer, M. Sarkar, J. Edmiston, R. Niessner, N. Pluym. "Assessment of the potential vaping-related exposure to carbonyls and epoxides using stable isotope-labeled precursors in the e-liquid". In: Nicotine & tobacco research (2021), Vol. 95 (8) pp. 2667-2676.

Contents

Glossary

2-HPMA	N-Acetyl-S-(2-hydroxypropyl)cysteine
3-HPMA	N-Acetyl-S-(3-hydroxypropyl)cysteine
AA	Acetaldehyde
ACR	Acrolein
AUC	Area under curve
CC	Conventional cigarettes
CI	Confidence interval
CNO	Cotinine-N-oxide
CO	Carbon monoxide
Cot	Cotinine
Cot-gluc	Cotinine-glucuronide
CRO	Crotonaldehyde
DHPMA	Dihydroxypropyl-mercapturic acid (metabolite of glycidol)
EC	E-cigarette
EI	Electron impact
ESI	Electrospray ioniziation
FA	Formaldehyde
G	Glycerol
GC	Gas chromatography
GC-MS/(MS)	Gas chromatography with (tandem) mass spectrometry
GLY	Glycidol
HEMA	N-Acetyl-S-(2-hydroxyethyl)-L-cysteine
HMPMA	N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine
HyPyBut	4-Hydroxy-4-(3-pyridyl)-butanoic acid
IS	Internal standard
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LLOQ	Limit of quantification
MA	Mercapturic acid
max	Maximum
min	Minimum
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTCA	2-Methyl-thiazolidine-4-carboxylic acid (metabolite of AA)
MTCG	2-Methyl-thiazolidine-4-carbonyl-glycine (metabolite of AA)
NAB	N-Nitrosoanabasin
NAT	N-Nitrosoanatabin
Nic	Nicotine
Nic-gluc	Nicotine-glucuronide
NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	N-Nitrosonornicotine
NNO	Nicotine-N-oxide
NorCot	Norcotinine
NorNic	Nornicotine

OH-Cot	trans-3'-Hydroxycotinine
OH-Cot-gluc	trans-3'-Hydroxycotinine-glucuronide
PAH	polycyclic aromatic hydrocarbon
PG	Propylene glycol
PO	Propylene oxide
QC	Quality control
SD	Standard deviation
SOP	Standard operating procedure
SPE	Solid phase extraction
TCA	Thiazolidine-4-carboxylic acid (metabolite of FA)
TCG	Thiazolidine-4-carbonyl-glycine (metabolite of FA)
TSNAs	Tobacco-specific nitrosamines
UPLC	Ultra-high pressure liquid chromatography
VOC	Volatile organic compound
W	Wattage

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1 Introduction

The use of e-cigarettes (e-cigs) has increased over the past years [1–4]. A reason for this increase might be the growing awareness on health by consumers since e-cigs are predominantly discussed as harm-reduced alternative to smoking and cessation aid by both the consumers and the public health community [5, 6]. This rising interest in e-cigs led to a controversial public health dispute [6–11]. There are a number of issues with the use of e-cigs (vaping) including: (i) Exposure of the active vaper or bystanders to toxicants, which are released or generated from the e-liquids during vaping thus increasing the health risk or exposed people [8, 9]; (ii) e-cigs may prevent smokers from quitting the smoking habit altogether; (iii) e-cigs may newly generate or maintain an existing addiction to nicotine [12]; (iv) young people may start vaping and then switch to conventional cigarettes (gateway effect) [13]; (v) e-cigs may make smoking socially acceptable again [14].

This thesis deals with the first issue of the above list. In particular, it was the aim of the investigations to characterize and quantify the internal exposure dose received by vapers under natural use (real life) conditions. The composition of the inhaled vapor and the absorbed dose is dependent on a variety of factors, including (a) the e-liquid composition, (b) coil temperature during vaporization of the e-liquid, (c) relative humidity, (d) ambient temperature (e) consumption of e-liquid per day, (f) vaping topography, (g) inhalation pattern.

Basically, e-liquids are composed 1,2-propylene glycol (PG) and/or glycerol (G) to which nicotine (0 – 20 mg/ml) and flavors have been added [13, 15–17]. These major e-liquid components also appear in the aerosol (vapor), are taken into the mouth and inhaled by the consumer. In many studies, a multitude of minor components including many toxicants in e-liquid and vapor of e-cigs have been identified and quantified [8, 17–20]. In a recent, comprehensive study, 150 chemicals were investigated in the aerosol of an e-cigarette with replaceable cartridges containing an e-liquid with tobacco aroma [20]. In total, 16 chemicals were identified to be generated by the e-cigarette, including the major components of the e-liquid (PG, G, nicotine), 3 nicotine impurities (myosmine, cotinine, NNN), 8 carbonyls and alcohols related to PG and G (allyl alcohol, formaldehyde, acetaldehyde, butyraldehyde, acrolein, glyoxal, methylglyoxal, acetone as well as chromium (probably from the heating coil) and chrysene (unknown origin).

The generation of carbonyls and other compounds from PG and G by heating has been studied by several authors [21–25]. Farsalinos [21, 22] observed formaldehyde in e-vapor only upon dry puff conditions, in which the e-liquid overheats. Sleiman et al. [25] reported the formation of following additional toxicants as thermal decomposition products from PG and G (in addition to those found by Margham et al. [20]): Propylene oxide, propenal, glycidol, acetol and diacetyl. The yields of these harmful chemicals increase upon increasing the voltage for vaping. Jensen et al. [23] performed NMR investigations with a single e-cig puff and could identify those PG/G-related chemicals mentioned above, as well as hydroxyacetone, dihydroxyacetone, lactaldehyde, glycolaldehyde and glyceraldehyde. Furthermore, this research group also observed the formation of relatively high amounts of hemiacetals of formaldehyde with PG and G upon high voltage vaping [23, 24, 26]. PG- and G-hemiformals are formaldehyde releasing agents. How these compounds behave in the respiratory tract, however, is unknown.

Apart from the device-related variables (points a and b above), vapers have available other, behavior-related variables (points c – e above), for varying and controlling the vaping (and nicotine) dose. As a common measure for daily e-cig use, the average e-liquid consumption per day is beginning to be established [27]. Variation in daily liquid consumption is in about the same range as the variation in daily cigarette consumption [28]. Furthermore, the vaping dose can efficiently be varied by topographic parameters such as puff volume, interval and duration [29–34]. For example, Kim et al. [35] in a review on e-cigs, reported averages from 5 studies of 51-118 ml/puff, 10-29.6 s and 1.8-4.3 s for puff volume, interval and duration, respectively. In a week long study of the topography of 20 e-cig users in their natural environment, high intra- and inter-individual variation in puff volume (mean $\pm$ SD: 65.4 $\pm$ 70.1 ml), flow rate (30.4 $\pm$ 11.4 ml/min) and duration (2.0 $\pm$ 1.8 s) was observed [33]. On average, vapers were observed to take larger and longer puffs compared to smokers of conventional cigarettes (CC) [36]. The inhalation pattern in vapers, in particular depth and duration, which are significant determinants of the absorbed dose have not yet been investigated systematically.

The considerations above suggest that dosimetry for e-cig users could most likely be achieved by measuring suitable biomarkers of exposure for the major constituents (PG, G and nicotine) and the other potential constituents formed under natural usage conditions in body fluids such as plasma, urine and saliva. The biomonitoring approach is assumed to be superior to measuring machine-derived emissions, since it takes into account the actual vaping behavior which decisively affects the absorption dose. A major problem with the biomonitoring approach is the fact that the chemicals of interest such as PG, G, nicotine, aldehydes, etc. have many sources and are not specific for the use of e-cigs.

To get around this problem, stable isotope-labeled major e-liquid constituents (PG, G and nicotine) could be applied. Stable isotopes have been commonly used to study dynamic metabolism. Studies using stable-isotope tracers were introduced in mass spectrometry as the “gold” standard method for understanding kinetics, uptake and distribution of various compounds in living organisms [37–43]. The advantage of this approach is that no interference from other sources (particularly diet and smoking of conventional cigarettes) would occur.

A clinical study was performed, in which experienced vapers used e-cigs stocked with e-liquids containing stable isotope-labeled PG, G and nicotine under both controlled and free (ad libitum) conditions. In this thesis, the performed clinical study is described together with the biomarker results for PG, G, nicotine and its major metabolites in various body fluids. Additionally, biomonitoring results for other aerosol potential constituents derived from PG and G (mainly carbonyls and epoxides) and nicotine (NNK and NNN) will be reported.

2 Fundamentals

2.1 E-Cigarettes

E-cigarettes are battery-powered vaporizers which simulate smoking without the combustion of the tobacco. The first e-cigarette was invented by Herbert A. Gilbert who patented "a smokeless non-tobacco cigarette" in 1965 [44]. This e-cigarette already consisted of a tank, a battery and had a cigarette-like shape (Figure 1). The tank could be filled with liquid, which could then be vaporized with the help of the battery. Despite the mature technology it never went into production. In the following decades further attempts were made to develop a combustion-free cigarette. These were mainly focused on the vaporization of nicotine and were not electronic in their nature.

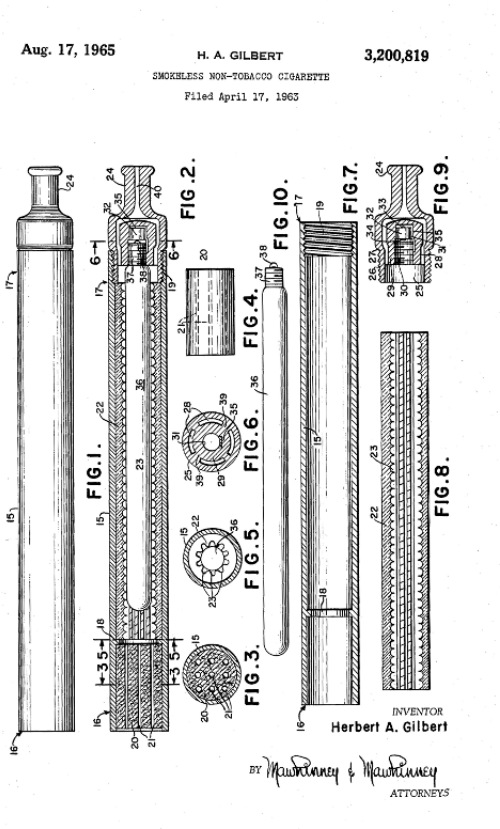


Figure 1: Scheme of the first e-cigarette invented by Herbert A. Gilbert [44]

In 2003 the idea of Gilbert was taken up again by the Chinese Hon Lik, pharmacist at the company "Golden Dragon Holdings" later "Ruyan Electronics". He developed the "first generation e-cigarette". This first model did not have the typical heating coil, which is needed to vaporize the liquid. Instead, he used a piezoelectric element that emitted ultrasonic waves. With the help of these ultrasonic waves, the liquid is vaporized. At that time, the liquid contained only propylene glycol and nicotine and was completely free of flavors. The use of a piezoelectric element was not suitable for mass production because of the size required. Therefore, Lik did further research and discovered that resistance heat gave a better result. This improved e-cigarette appeared on the Chinese market in 2004 and was exported worldwide in 2005. Due to the lack of a patent until 2007, it was copied and further developed by various companies. Until 2007, e-cigarettes were one unit.

In 2006/2007 the British company Umer and Tariq Scheikh developed the "Cartomizer". The "Cartomizer" is a combination of vaporizer and depot which can be placed directly on the battery carrier of the e-cigarette. The tank, in which the e-liquid is stored, is filled with cotton wool. The cotton wool in the depot absorbs the liquid and then slowly releases it to the vaporizer and the heating coil inside. So the highlight of the "Cartomizer" was that the heating coil was integrated directly into the liquid container. Due to the cotton wool in the depot, the level of the liquid could not be seen, which could lead to problems like the "Dry Puff". The "Dry Puff" is a very unpleasant and health-dangerous experience for the e-cigarette user, because it tastes like burnt cotton wool and it includes a high amount of thermal degradation products. To

solve this problem the "Clearomizer" was developed. The Clearomizer does not use cotton, making the tank transparent (hence the name "clear") and allowing the level of the e-liquid to be closely monitored. Thus a puff without liquid ("Dry Puff") can be avoided. This type of e-cigarette is the third generation of e-cigarettes which has still the look of a cigarette. The fourth and latest generation looks more like a box. The fourth generation of e-cigarettes (Figure 2) is characterized by stronger batteries, larger tanks, higher vaporizers as well as more extensive adjustment possibilities (e.g.: the power, temperature). From this development process three main categories are on the market: (i) cigalikes, which resemble conventional cigarettes in shape and size; (ii) eGos, which are larger than cigalikes, usually with a removable tank system which can be refilled with e-liquids; and (iii) mods, which are almost endlessly customizable and larger than eGos [45].

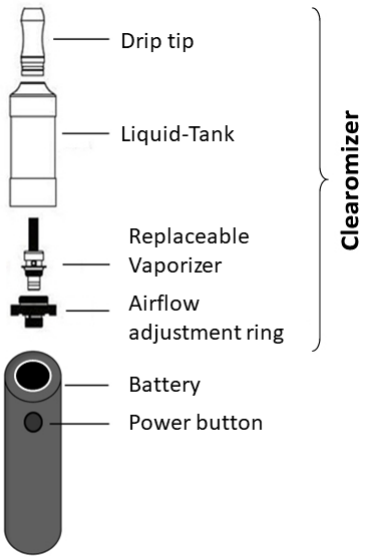


Figure 2: Scheme of an e-cigarette of the fourth generation

The e-cigarette used in this study belongs to the second group, the so called "eGos". The e-cigarette consists of the rechargeable battery and the clearomizer. The clearomizer is divided into a airflow adjustment ring, a replaceable vaporizer, a clear liquid tank and a drip tip. The airflow adjustment ring regulates the inhaled air resistance to the vapor. The device has four different sized airflow holes with a diameter of 0.9 mm, 1.1 mm, 1.4 mm or 1.8 mm. The larger the diameters is, the more air can flow into the device and the drawing resistance is lowering. Above the flow adjustment ring is the replaceable vaporizer. The replaceable vaporizer is a bottom vertical coil (BVC). BVC means that the coil runs vertically from bottom to top. The coil is a thin wire around which the cotton wool is wrapped. Around the vaporizer is the clear liquid tank which consists of Pyrex glass. The e-liquid is placed in the liquid tank where it is absorbed by the cotton wool. The cotton-wrapped wire transforms the applied current from the battery module into heat through its resistance. This heat evaporates the liquid that is in the cotton and forms so the aerosol. The amount of the generated aerosol can be adjusted by the power settings.

2.1.1 Composition of the e-liquid

E-liquids are a mixture of four basic components: The diluent (consisting usually of the ingredients propylene glycol and glycerol), nicotine, flavor compounds and water [13, 15–17]. The composition of propylene glycol and glycerol can vary depending on whether a liquid is propylene glycol-based (larger proportion of propylene glycol) or glycerol-based (larger proportion of glycerol). The maximum nicotine concentration in the liquid in Europe is according to Tobacco Products Directive (2014/40/EU) 2 mg/ml [46], whereas there are no restriction in the United States. The added flavors vary from tobacco-like flavors over the fruity flavors to biscuit flavors (like straw-

berry cheese cake and gingerbread). In 2014, a study counted 7764 unique flavors from 466 different brands [47]. There are a large variety of chemicals which are used to generate the different tastes (e.g.: 2,3-pentanedione , isobutyl acetate, ethyl butyrate, butyl butyrate, isoamyl acetate, 2,3-dimethylpyrazine, 3-methyl-1-butanol, limonene, 2,3,5-trimethylpyrazine, benzaldehyde, (Z)-3-hexen-1-ol, menthol, 2-acetylpyrrole, benzyl alcohol, methyl salicylate, cinnamaldehyde, methyl anthranilate, (+)-aromadendrene, cinnamyl alcohol, methyl cinnamate, maltol, ethyl maltol, and coumarin).

Propylene glycol (propane-1,2-diol) is a diol alcohol. It is a hygroscopic, tasteless, odorless and colorless clear oily liquid. Due to its various miscibility with other solvents (e.g.: water, acetone, chloroform) it is widely used in industry (e.g.: cosmetic industry as preservative, humectant and spreader [48]; food industry to prevent fermentation and mold growth [49–51]; intravenous, oral, topical pharmaceutical preparations; chemical industry[48, 52, 53], ingredients of e-cigarettes). After absorption, propylene glycol is metabolized to carbon dioxide and water through metabolic intermediates including lactic and pyruvic acids. In addition, a portion of the drug is excreted unchanged via the renal pathway [54, 55].

Glycerol (propane-1,2,3-triol) is a triol alcohol. It is a colorless, odorless, viscous liquid which has a sweet taste. Glycerol is widely used in the food industry as sweetener [56, 57] and humectant [58] and in pharmaceutical formulations [59–61]. It has also an important role in triglyceride formation. The three-carbon substance forms the backbone of the triglycerides where the fatty acids react with the hydroxy-group of the glycerol [59]. When the body uses stored fat as source of energy, glycerol and fatty acids are released into the bloodstream. Glycerol can be converted by the liver into glucose which provides energy for the cellular metabolism, so called gluconeogenesis. Another metabolic pathway is the glycolysis where glycerol is metabolized to pyruvate. The amounts of unchanged glycerol in urine depend on the uptake of glycerol [62, 63]. Glycerol shows very little acute toxicity and very high oral doses or acute exposures can be tolerated.

Nicotine is an alkaloid which is naturally produced in the nightshade family of plants, like potatoes, tomatoes, eggplants and tobacco [64]. Apart from nicotine uptake by tobacco, nicotine uptake by the other nightshade plants is of minor importance [64]. It has a pale yellowish, slightly fish-smelling, oily liquid. Nicotine acts as a receptor agonist at nicotinic acetylcholine receptors except for two nicotinic receptor subunits where it acts as an antagonist [65]. Because of these properties nicotine has a stimulating effect and is a parasympathetic drug. Nicotine is addictive [66, 67] and toxic [68]. It is mainly used in medicine [69], as pesticide [70, 71] and as recreational drug including cigarettes, chewing tobacco, e-cigarettes, snuff etc. [67, 72]. Nicotine is diversely metabolized by different enzymes including cytochrome P450 enzymes, aldehyde oxidase, flavin-containing monooxygenase 3, amine N-methyltransferase, and UDP-glucuronosyltransferases [73]. The metabolites and nicotine occur in various matrices, like plasma, urine, saliva and sputum [74–80].

2.1.2 Composition of the aerosol

An aerosol is a collection of solid or liquid particles suspended in gas [81]. Hence, they are a two-phase system (e.g. dust, fume, smoke, mist ,fog, haze clouds, and smog). Therefore, the two matrices of interest in this work are aerosols: Vaporized e-liquid by an e-cigarette and mainstream smoke of the conventional cigarette.
In this work, "aerosol" will refer to the vaporized e-liquid of the e-cigarette. Whereas, the main stream smoke of the cigarette will be refereed as "smoke".
The three substances described in Section 2.1.1 are combined with the flavor chemicals in various proportions to produce the e-liquid. By heating these e-liquids in the e-cigarette an aerosol is formed. Depending on the power and the resulting temperature, other thermal decomposition products may be formed in addition to the main components propylene glycol, glycerol, nicotine and flavor chemicals (see Section 2.1.3). The aerosol with the substances obtained is absorbed through the mucous membranes of the respiratory tract. The amount of uptake is highly dependent on the user, the e-cigarette and the vaping conditions (puff duration, generated amount of aerosol).
The generated aerosols are collections of droplets surrounded by a gas phase [82]. The distribution of each substances between the gas and the droplet phase depends on their volatility, the type of the solvent, temperature and the pH-value. In polar substances, like propylene glycol and glycerol, hydrophilic substances, like nicotine, tobacco specific nitrosamines, are more likely to be solved in the particle phase [83]. Whereas hydrophobic substances, like propanal, formaldehyde, benzene are more likely to be in the gas phase [84]. The compound dependent equilibrium gas/particle coefficient $K_{p,i}$ is defined as [84]:

$$K_{p,i}[m^3/\mu g] = \frac{c_{p,i}[\mu g/\mu g]}{c_{g,i}[\mu g/m^3]} \tag{1}$$

$$K_{p,i}[m^3/\mu g] = \frac{RT}{10^6 \overline{\mathrm{MW}} \zeta_i p^{\circ}_{L,i}} \tag{2}$$

$c_{p,i}[\mu g/\mu g]$: concentration in the particle phase

$c_{g,i}[\mu g/m^3]$: concentration in gas phase

R $\left[\frac{m^3 atm}{molK}\right]$: gas constant $\left(8.2 \cdot 10^{-5} \frac{m^3 atm}{molK}\right)$

$T[K]$: Temperature

$\overline{\mathrm{MW}}\left[\frac{g}{mol}\right]$: average molecular weight of the absorbing liquid phase

ζ_i: mol-fraction-scale activity coefficient in the liquid (L) phase

$p^{\circ}_{L,i}[atm]$: vapor pressure of pure liquid at temperature T

Although the formation of a complete equilibrium during a puff is unlikely, this coefficient serves as an indication of the distribution of the substances under investigation in the gas and particle phase [85].
Therefore different methods for determination of the substances of interest were developed taking into account the distribution in gas and particle phase, respectively. Additionally, the uptake of these substance were monitored through the different biomarkers.

2.1.3 Thermal decomposition products

Thermal decomposition (thermolysis or pyrolysis) is a chemical decomposition caused by heat [86]. The reaction can be either endothermic or exothermic (e.g.: oxidation) to break the chemical bond. The decomposition temperature of a substance is substance-dependent and defines the temperature at which the substance chemically decomposes.

Thermal decomposition of the components in the e-liquid (PG, G, nicotine, water and flavors) can occur at high power or high temperature, respectively. The aerosol is formed by heating the e-liquid. During this process the constituents may be decomposed into toxicants such as carbonyls, epoxides or nitrosamines. The formation highly depends on the type of e-cigarette [87], the applied power/temperature [88, 89] and the vaping behavior of the user [90]. The amount of the formed thermal decomposition products is diversely discussed in the literature [20, 22, 25, 30, 42, 91–100]. A growing number of studies have also investigated the emissions of some cigarette smoke toxicants from e-cigarettes, such as tobacco-specific nitrosamines [101, 102], tobacco alkaloids and nicotine decomposition products [103] , volatile organic compounds [104, 105], aromatic amines [106], carbon monoxide [106], polycyclic aromatic hydrocarbons (PAHs) [107] phenolics [102], metal compounds [108] and carbonyls [23, 87, 89, 96, 98, 109, 110]. Most of these studies report aerosol emission levels of toxicants that are either undetectable or a few percent of those found in cigarette smoke, and comparisons have also been made to room air [106]. Nevertheless, there also studies which state that there are higher amounts of thermal decomposition products, especially concerning the carbonyls [22].

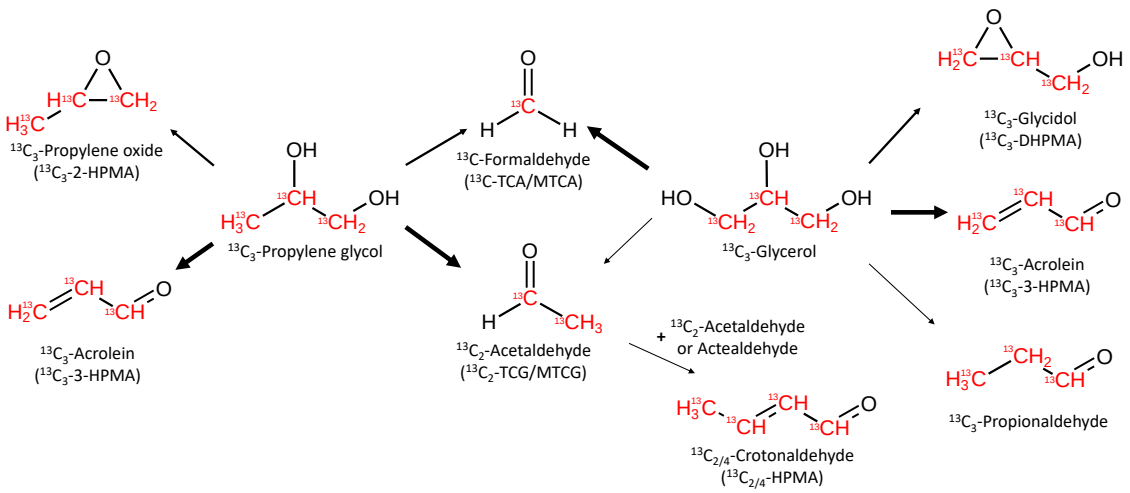


Figure 3: Overview of the thermal degradation products formed from $^{13}C_3$-propylene glycol ($^{13}C_3$-PG) and $^{13}C_3$-glycerol ($^{13}C_3$-G). The thickness of the arrows represent the contribution of $^{13}C_3$-PG and $^{13}C_3$-G to the formation of the degradation products ^{13}C-formaldehyde ($^{13}C_3$-FA), $^{13}C_2$-acetaldehyde ($^{13}C_2$-AA), $^{13}C_3$-acrolein ($^{13}C_3$-ACR), $^{13}C_3$-propionaldehyde ($^{13}C_3$-PA), $^{13}C_{2/4}$-crotonaldehyde ($^{13}C_{2/4}$-CR), $^{13}C_3$-glycidol ($^{13}C_3$-GLY), and $^{13}C_3$-propylene oxide ($^{13}C_3$-PO) according to Sleiman et al. and Uchiyama et al. [25, 98]. The corresponding biomarkers ^{13}C-((methyl-)thiazolidine carboxylic acid (^{13}C-(M)TCA), ^{13}C-(methyl-)thiazolidine carbonyl glycine (^{13}C-(M)TCG), $^{13}C_3$-2,3-dihydroxypropylmercapturic acid ($^{13}C_3$-DHPMA), $^{13}C_3$-3-hydroxypropylmercapturic acid ($^{13}C_3$-3-HPMA), $^{13}C_3$-2-hydroxypropylmercapturic acid ($^{13}C_3$-2-HPMA), $^{13}C_{2/4}$-hydroxymethylpropylmercapturic acid ($^{13}C_{2/4}$-HMPMA) are shown in brackets.

Volatile organic compounds (VOCs) such as the aldehydes formaldehyde (FA), acetaldehyde (AA), acrolein (ACR), or the epoxides propylene oxide (PO) and glycidol are of particular interest in the risk assessment of e-cigarettes due to their possible formation from G and PG in the aerosol according to Figure 3. The thermal degradation of PG and G in aerosol from e-cigarettes was recently investigated by Sleiman et al. [25] and Melvin et al. [41]. These authors reported the decomposition of glycerol to glycidol and acrolein, while acetaldehyde, formaldehyde, methyl glyoxal, propylene oxide, and propanal were formed from propylene glycol (Figure 3). Moreover, it is postulated that crotonaldehyde (CRO) may be generated by aldol condensation of acetaldehyde. Lastly, ethylene oxide may also be formed during pyrolysis. The aforementioned VOCs are constituents of the gas phase of cigarette smoke but also originate from various other environmental and endogenous sources resulting in high background levels, especially for FA and AA [111–118]. Reported FA and AA concentrations in e-cigarette aerosol highly vary depending on the used e-cigarette but also on the methodology and issues with background levels are frequently discussed [22, 88, 109]. Hence, introducing the stable-isotope labeled constituents $^{13}C_3$-PG and $^{13}C_3$-G into the e-liquid as potential precursors helps to unequivocally assess the formation of VOCs specific to the use of the electronic e-cigarettes.

Additionally, the presence of the nicotine-derived carcinogens N-nitrosonornicotine ketone (NNK) and N-nitrosonornicotine (NNN), possibly formed either under vaping conditions or by endogenous nitrification can also be assessed through this approach by measuring the corresponding labeled biomarkers in urine.

2.2 Stable-isotope labeled precursors

Isotopes are atoms with the same number of protons but different numbers of neutrons. They have the same atomic number and therefore represent the same element, but have different mass numbers. There are stable and unstable isotopes. Unstable isotopes are transformed into other elements by radioactive decay according to their substance-specific half-life.

Stable isotopes (e.g.: oxygen, hydrogen, sulfur, nitrogen and carbon) are used exemplarily in geochemistry, analytical chemistry and kinetic studies. In the mass spectrometry, the stable-labeled isotopes (e.g.: labeled with ^{2}H = D or ^{13}C) have an important role. They are mainly used as internal standards, but also as the "gold" standard method in clinical studies for understanding kinetics, uptake , distribution, metabolism and elimination of various compounds in living organisms [37–39, 43].

For avoiding interference from PG, G and N and/or their pyrolysis products taken up from other sources (diet, environment, tobacco products, etc.) a certain percentage of the major e-liquid constituents was replaced by stable-isotope labeled analogues. The suitable percentage for the described purpose was estimated to be 10 % of the originally present amount of PG, G and N in the e-liquid. The applied labeled replacement compounds are shown in Figure 4.

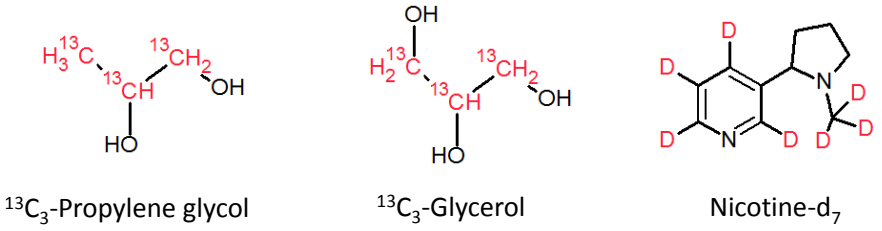


Figure 4: Labeling of the main ingredients of the e-liquid used in this study

Using these labeled components an e-cigarette-specific uptake of the main constituents and the formation of metabolites as well as the uptake and metabolism of potentially toxicants can be deduced. In order to show that the applied methodology is capable of assessing the pyrolytic formation of the toxicants of interest from the labeled precursors, a positive control was introduced in the experimental approach. Smokers, smoking CC spiked with labeled PG, G and N, were considered as suitable positive control, since the burning conditions in a cigarette make the pyrolytic/pyrosynthetic formation of labeled FA, AA, ACR, CRO, ethylene oxide, propylene oxide, glycidol, as well as NNK and NNN highly likely. Therefore, the analytical detection of labeled metabolites of these toxicants (or at least part of them) formed during combustion (1) are regarded as proof of concept of the scientific approach.

The work with labeled and same unlabeled components simultaneously raises the problem of the isotopic overlap. The influence of the isotopic overlap depends on the used labeling and mass difference between the unlabeled and the labeled. Therefore the results of the labeled component has to be corrected, the so called isotopic correction. The procedure of isotope correction was performed according to Scherer et al. [119]. The procedure of the isotopic correction will be explained exem-

plarily for TCA (the remaining metabolites are summarized in Appendix E). The mass transitions for TCA (m/z 262 $>$ 174) and ^{13}C-TCA (m/z 263 $>$ 175) differ only by a single mass unit. Given the fact that the established chromatography provides co-elution of both isomers, isotope correction needs to be considered to achieve accurate results. In this case, the isotopic overlap derived from the charged fragment ion of unlabeled TCA needs to be considered. The isotope distribution for this analyte can be described as follows: ^{13}C (1.07 %), ^{2}H (0.015 %), ^{15}N (0.37 %), ^{17}O (0.0385 %) and ^{33}S (0.75 %). The theoretical contribution of this fragment to the signal intensity of the ^{13}C-TCA was calculated at 9.1 %. In order to validate this approach, the isotopic overlap was determined experimentally. Therefore a calibration curve with unlabeled TCA was analyzed with respect to the overlap to ^{13}C-TCA. The overlap was determined by measuring both mass transitions, labeled and unlabeled. The area of the ^{13}C-TCA was divided by the area of the ^{12}C-TCA and the ratio was determined as the experimental isotopic overlap over the whole concentration range. I found excellent agreement between the theoretical overlap calculated value of 9.1 % by Analyst 1.6.3 (SCIEX, Darmstadt, Germany) and the overlap experimentally determined value of 8.8 %.
Thus, for an accurate quantification of the ^{13}C-labeled biomarkers, the isotopic overlap needs to be considered by using the following equation:

$$L_{corrected} = L - U \cdot A \tag{3}$$

$L_{corrected}$: corrected area ratio of the labeled fragment
L: area ratio of the labeled fragment
U: area ratio of the unlabeled fragment
A: calculated percentage of the isotopic distribution

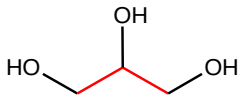
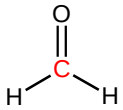
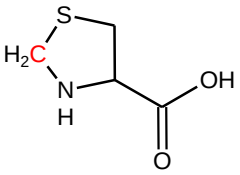
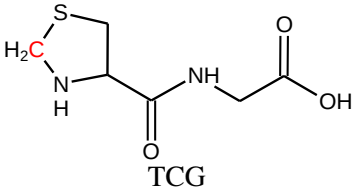
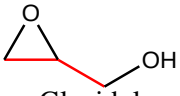
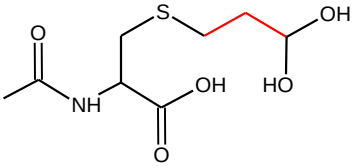
The A-values of the other analytes are summarized in Appendix E in Table 33. The experimental A-values were used for the isotopic correction in this thesis.

2.3 Biomarker

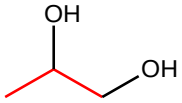
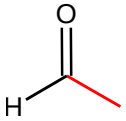
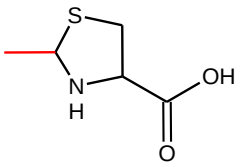
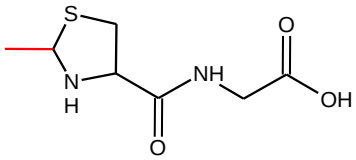
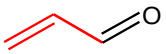
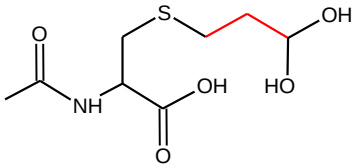
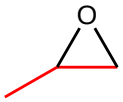
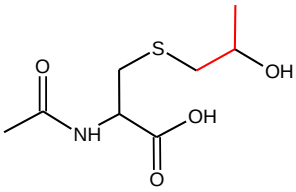
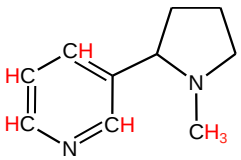
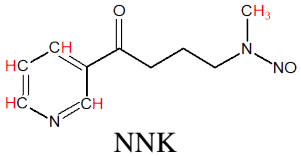
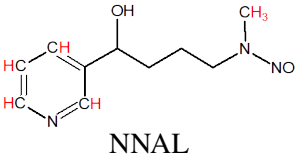
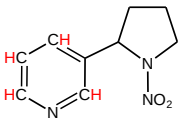
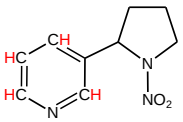
Biomarkers are specific biological characteristics that can be used as a reference for processes and disease states in the body. Therefore, they are of great interest for the biomonitoring. In the biomonitoring, biomarkers are divided into two different categories, the biomarker of exposure and the biomarker of effect. Biomarkers of exposure are chemicals or their direct metabolites, determined after exposure to the substances in the body. Biomarkers of effect originate from endogenous pathways that are altered by an exogenous effect. As a result, the response time is much longer compared to biomarker of exposure.

For answering the questions of this work, the focus was on the biomarker of exposure. The screening of these biomarkers can be performed in various body fluids. Urine, plasma, saliva and sputum were collected in this study. Urine and saliva are non-invasive biomarkers which increases the willingness and the compliance of the subjects to participate. Unfortunately, the entire biomarker profile is not reflected in saliva and urine. For this reason plasma, as an invasive matrix, has to be investigated additionally. Furthermore, sputum was considered as an interesting matrix to investigate the different biomarkers in the lung of the smoker and the vaper.

Table 1: Biomarkers of exposure to toxicants, which (theoretically) may be formed from the major precursors in e-liquid PG, G and N. The common carbon backbone in the precursors, toxicants and biomarkers are shown in red. ΔT: thermal decomposition by high temperature; ((methyl-)thiazolidine carboxylic acid ((M)TCA);(methyl-)thiazolidine carbonyl glycine ((M)TCG); 2,3-dihydroxypropylmercapturic acid (DHPMA); 3-hydroxypropylmercapturic acid (3-HPMA), 2-hydroxypropylmercapturic acid (2-HPMA), hydroxymethylpropylmercapturic acid (HMPMA).

Precursor in e-liquid	Hypothetical mechanism	Toxicant formed	Biomarker for the toxicant
 Glycerol	ΔT	 Formaldehyde	 TCA  TCG
	ΔT	 Glycidol	 DHPMA

Continuation of Table 1: Biomarkers of exposure to toxicants, which (theoretically) may be formed from the major precursors in e-liquid PG, G and N.

Precursor in e-liquid	Hypothetical mechanism	Toxicant formed	Biomarker for the toxicant
 Propylene glycol	ΔT	 Acetaldehyde	 MTCA  MTCG
	ΔT	 Acrolein	 3-HPMA
	ΔT	 Propylene oxide	 2-HPMA
 Nicotine	ΔT	 NNK	 NNAL
	ΔT	 NNN	 NNN

The underlying assumptions for the analysis of biomarkers of exposure to toxicants potentially derived from precursors in the e-liquid are shown in Table 1. In general, thermal decomposition (pyrolysis, ΔT) of PG and G may lead to a series of toxicants (mostly carbonyls and epoxides), which in turn are metabolized after uptake, leading to the formation of the biomarkers. In case of nicotine, the formation of NNK (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N-Nitrosonornicotine) by a pyrosynthetic process is possible. The corresponding biomarkers NNAL

(4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol) and NNN in urine could unequivocally show prove or disprove whether pyrosynthesis or endogenous formation takes place.

2.3.1 Nicotine and its metabolites

Nicotine is an alkaloid which occurs in the nightshade family plants. One of the most popular nightshade plants is tobacco [120]. Tobacco is one vehicle for the absorption of nicotine in the human body by smoking cigarettes [67]. The absorbed amounts and the metabolism is well examined for the smokers [121–123]. Additional nicotine delivery products are released on the markets [69], like nicotine sprays [124, 125], transdermal patches [126] or the e-liquids [16, 72]. Most recent e-liquid entered the public markets. The e-liquids are heated by using an e-cigarette and form an aerosol. The composition of the aerosol varies depending on the e-cigarette, e-liquid and the user. Therefore, the uptake of nicotine upon vaping (e-cigarette use) depends on the amounts in the e-liquids as well as on the vaping pattern and experience of the user [30, 127, 128]. To clearly assign the nicotine uptake to vaping, the e-liquid was spiked with deuterated nicotine in this study.

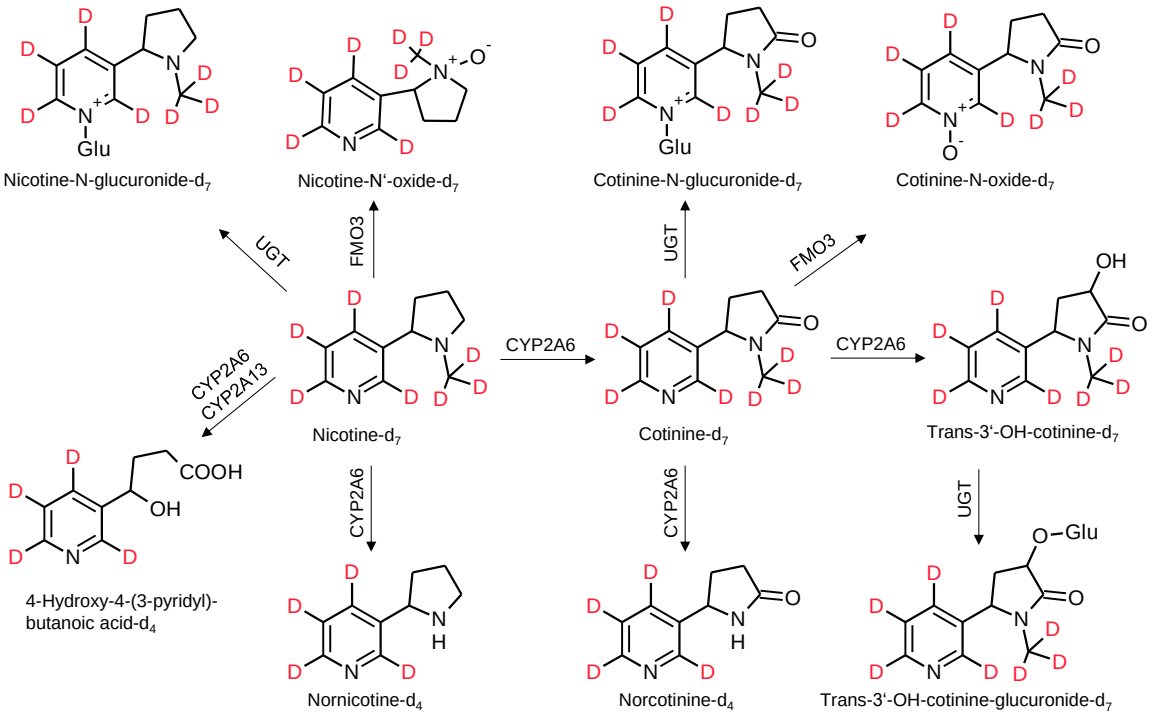


Figure 5: Metabolism of labeled nicotine and its major urinary metabolites according to [74]. CYP: Cytrochrome P450; FMO: Flavin-containing monooxygenase; UGT: Uridin-5'-diphospho-glucuronosyltransferase.

Nicotine is primarily metabolized by the cytrochrome P450 2A6 (CYP2A6) which is a liver enzyme [129]. Furthermore, nicotine is metabolized by N-oxidation (primarly by FMO3) forming nicotine-N-oxide; by glucuronidation (mainly by UGT2B10) resulting in nicotine-N-glucuronide and some other enzymes which contribute less to the nicotine metabolism [73]. Cotinine is the major metabolite of nicotine. Cotinine is further metabolized by the same enzymes as nicotine forming for instance

hydroxycotinine, cotinine-N-oxide, cotinine-N-glucuronide. The detailed metabolism of the labeled nicotine is shown in Figure 5 including the participating enzymes.
The formation of hydroxycotinine from cotinine involves exclusively CYP2A6 [130]. The catalytic activity of CYP2A6 displays large inter-individual differences due to polymorphism in the CYP2A6 gene [131]. The polymorphism occurs by different mutations like point mutation and gene deletion. These nucleotide changes show a high inter-ethnic variability resulting in different phenotype groups: (i) 'poor' metabolizer (no active CYP2A6 alleles, homozygous for inactive alleles), (ii) 'slow' (one inactive or two decreased activity alleles), (iii) 'intermediate' metabolizer (heterozygous with one decreased activity allele and one active allele), (iv)'normal'/ 'extensive' (2 active alleles), or (v) 'ultrarapid'/ 'fast' (>2 active alleles) [132–136]. As a measure of CYP2A6 enzymatic activity, the ratio between 3-hydroxycotinine and cotinine is used. This ratio is known as nicotine metabolite ratio (NRM) [137, 138].

2.3.2 Tobacco specific nitrosamines

Tobacco-specific nitrosamines (TSNAs) are a component of young tobacco leaves [139–142]. However, in the leaves are only traces of TSNAs. They are mainly formed by nitrosation of the alkaloids in tobacco, for example nicotine, anatabin and anabasin. The formation of TSNAs is largely due to different drying and fermentation processes of raw tobacco [139, 143, 144]. Nitrosamine levels also increase as tobacco is burned. In addition, they may be produced via endogenous nitrosation processes in the human body [145, 146].

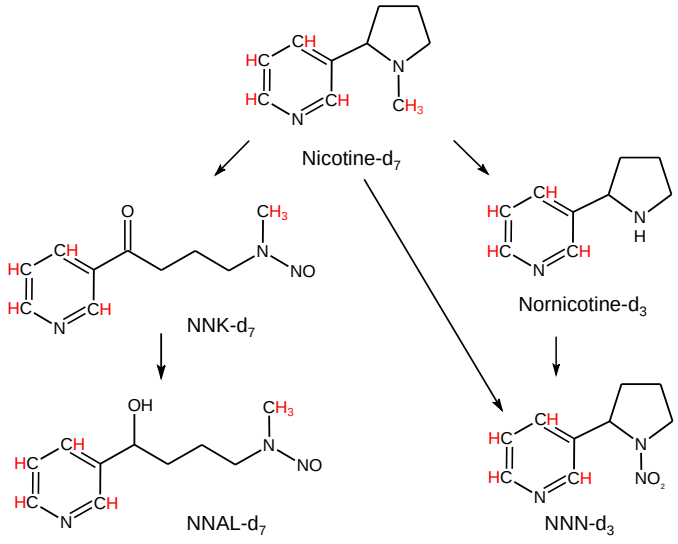


Figure 6: Formation of NNK (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone), NNAL (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol) and NNN (N-Nitrosonornicotine) from labeled nicotine. Adapted from [147]. The red colored "H" represent the position of the deuterium.

Due to the presence of nicotine in the e-liquids, tobacco-specific nitrosamines (TSNAs) might be found in the e-liquid and aerosol [22]. The formation of the TSNAs from labeled nicotine is shown

in Figure 6. These compounds represent one of the most carcinogenic groups in the tobacco products, so the present in the aerosol of an e-cigarette or in the e-liquid is crucial for any risk assessment. The evaluation of TSNAs was restricted to NNK (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N-Nitrosonornicotine). These two TSNAs are directly formed from nicotine. They can unequivocally be traced back to the nicotine from the e-liquid due to the labeled nicotine. Additionally NNAL (4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol) was evaluated in the urine sample because NNAL is the biomarker of NNK.

2.3.3 Mercapturic acids

Mercapturic acids are the condensation products of N-acetyl-cysteine with electrophilic compounds (Figure 7), such as carbonyles or epoxides . They are formed in the liver and excreted in urine. Endogenous or exogenous electrophilic compounds (EC) are (1) absorbed by the cells. The following conjugation with glutathione (GSH) is catalyzed by glutathione S-transferases.

The generated GSH-conjugate (EC-[Glu-Cys-Gly]) is (3) transported to the extra-cellular space by ATP-binding proteins. In the extra-cellular space the (4) glutamate and (5) glycine residues can be hydrolyzed by the membranebound extracellular enzymes (4) glutamyl transpeptidades (GGT) and (5) dipeptidases (Pept.), forming cysteinyl-glycine (EC-[Cys-Gly]) and cysteine-conjugates. The newly formed cysteine-conjugate are (6) reabsorbed by the cell which depends on multiple importers (Impt, e.g., organic anion transporting polypeptides and cystine/cysteine exchangers). In the cell, the cysteine-conjugate is (7) N-acetylated by N-acetyltransferases, generating the final mercapturic acid N-acetylcysteine-conjugate (EC-[NAC]). (8) The mercapturic acid is further transported to the extracellular space by exporter proteins (Expt., e.g., organic ion transporter 1), and excreted from the organism.

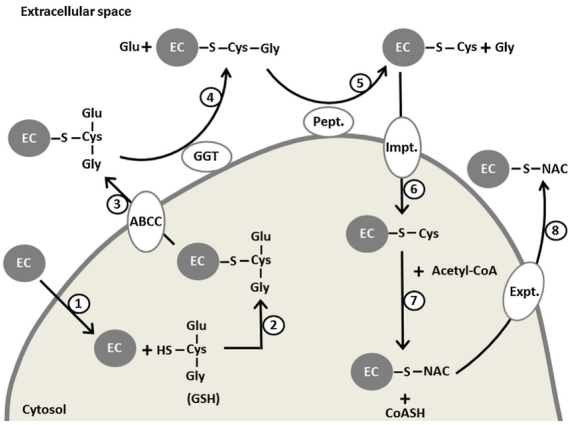


Figure 7: Scheme of the formation of mercapturic acids in cells from Ramsay et al. [148]. For illustrative reasons the process is shown in a single cell.

This work focused on the determination of mercapturic acids of propylene oxide (2-HPMA), acrolein (3-HPMA), glycidol (DHPMA), crotonaldehyde (HMPMA), and ethylene oxide (HEMA) in urine since they could be possible formed from the precursors PG and G (cf. Section 2.1.3).

2.3.4 Biomarkers of formaldehyde and acetaldehyde

Formaldehyde (FA) is classified as carcinogenic to humans (Group 1) and acetaldehyde (AA) as possibly carcinogenic to humans (Group 2B) by the International Agency of Research on Cancer (IARC) [149, 150]. FA is an extremely reactive chemical that interacts with various functional groups, such as primary and secondary amino [151], hydroxyl, thiol and amido moieties of biological macro-

molecules. This can lead to the formation of DNA, RNA and protein adducts as well as crosslinks [152]. Except for crosslinking, AA chemically reacts in a similar way.

FA and AA are widely used as basic chemicals or as intermediates in the chemical industry. In addition FA is frequently used as disinfectant and preservative [153–155]. Apart from its use in the chemical industry, FA occurs as endogenous compound in most living organisms [111–113]. Concentrations of FA in outdoor air range from 0.001 (remote and rural areas) to 0.02 mg/m^3 (urban areas) [114]. Indoor air FA concentrations are even higher and usually range from 0.01 to 0.1 mg/m^3 [115]. AA is a product of combustion of organic material and photo-oxidation of hydrocarbons in the atmosphere [116, 117]. Furthermore, AA is an intermediate product in the metabolism of ethanol and sugars [118]. Therefore, suitable biomarkers of exposure to FA and AA are of great interest. However, given their almost ubiquitous occurrence makes the biomonitoring of FA and AA a challenging task with respect to attribution to specific sources of exposure.

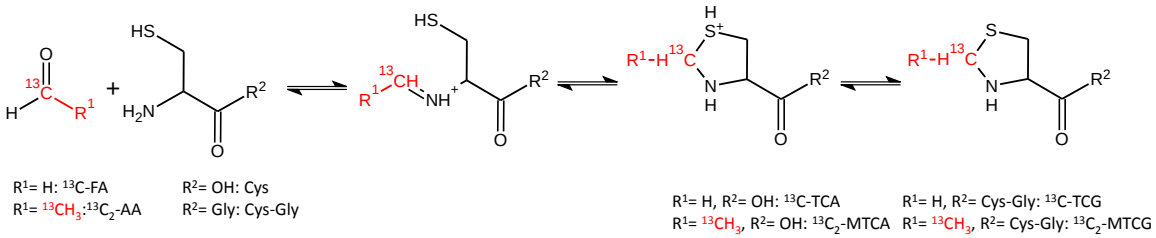


Figure 8: Formation of the labeled (methyl)-thiazolidine carboxylic acid (M)TCA and of the labeled (methyl)-thiazolidine carbonylglycine (M)TCG in the human body by cyclization of FA and AA, respectively, with Cys(-Gly) [156–158]

FA and AA are rapidly metabolized but can also react virtually instantly with nucleophiles in the organism [118, 159–163], yielding reaction products suitable for biomonitoring. Frequently used biomarkers are formate and acetate [164–168]. Analytical methods for these markers include GC-MS methods with or without derivatization prior to analysis [166, 167, 169] as well as enzymatic assays [170]. Large intra- and inter-individual variance in the physiological levels of these metabolites limits their suitability as biomarkers of exposure [115, 171]. Formate is involved in a number of endogenous processes [172–174]. Thus, the exposure to formaldehyde may influence the formate body concentration only to a small extent, rendering formate useless as a biomarker of exposure to formaldehyde. Acetate is consumed during the citric cycle (in the form of acetyl-CoA) [175] and produced during hydrolysis of acetylated metabolites. Due to these different influences on acetate and formate concentrations in blood and urine, they are not suitable as biomarkers of exposure to these aldehydes. Sulfur containing metabolites have been described as specific biomarkers for the exposure to formaldehyde and acetaldehyde [156–158, 176–178]. These sulfur containing metabolites are reportedly formed by a non-enzymatic condensation reaction of FA/AA with cysteine [156–158, 176, 177] or the glutathione metabolite cysteinylglycine (Figure 8) [178]. The cyclization reaction results in the biomarkers 4-thiazolidine carboxylic acid (TCA) and 4-thiazolidine carbonylglycine (TCG) for FA as well as 2-methyl-thiazolidine-4-carboxylic acid (MTCA) and 2-methyl-thiazolidine-4-carbonylglycine (MTCG) for AA. The occurrence of MTCA, as biomarker of acetaldehyde, was

already observed after ethanol consumption [157], overcoming the high variability of acetate. However the high endogenous and exogenous levels of FA and AA remain a problem. This prompted me to develop an analytical method for the systematic evaluation of the absorption of FA and AA into the human body by cigarette smoking and e-cigarette vaping, respectively. The approach using $^{13}C_3$-labeled PG and G should be able to overcome the high endogenous and exogenous levels of FA and AA.

2.4 Analytical methodologies

During this thesis it was essential to adapt existing methods for the stable-isotope labeled precursors, their labeled thermal decomposition products and the resulting metabolites as well as to develop new methods for the missing analytes. The calibration was always conducted with the unlabeled analyte. If the analyte was available in the labeled form, it was checked whether the linearity of calibration between the labeled and unlabeled analyte matched. For the tested analytes the slope of the calibration curve fitted well together for the unlabeled and the labeled analyte.
The complexity of the different matrices (body fluids, aerosol, smoke and e-liquid) as well as the quantification of labeled alongside with unlabeled compounds needed a combination of separation technology with a sensitive and selective detection technology. Tandem mass spectrometry combined with liquid or gas chromatography was the methodology of choice for these analysis. The liquid and gas chromatography, respectively, offered the possibility to separate the analytes from the other matrix components by using different solid and mobile phases (liquid or gas phase). Whereas the tandem mass spectrometry offers the mass selective detection of the different analytes and through the different fragmentation pattern of each analyte a good specificity. Therefor the analysis were mainly conducted with tandem mass spectrometry combined with liquid chromatography (LC-MS/MS) and mass spectrometry combined with gas chromatography (GC-MS).
In the present research project, the available methods were modified so that the labeled biomarkers could be also measured. Furthermore, the expected lower levels of labeled compounds in body fluids required a significant increase in the sensitivity of the analytical methods.

2.4.1 Adaption

The methods based on validated in-house methods. For the adaption of these methods, the fragmentation pattern for the analytes had to be elucidated. There were two different approaches: (i) the labeled compound was available; (ii) the labeled analyte was not available such as some of the metabolites or decomposition products. If the compound was available, the analyte has been tuned by infusion to the mass spectrometer. If the analyte was not obtainable, the fragmentation pattern was resolved based on the literature. For the following analytes was an in-house method available:

- Nicotine and its 10 metabolites in urine
- Tobacco specific nitrosamines
- Mercapturic acids
- Creatinine

2.4.2 Development

The required methods were developed in different matrices, like bodyfluids, aerosol and e-liquid. Therefore different sample preparation procedure, as well as different instruments were needed. Due to the simultaneous determination of labeled and unlabeled analytes, mass spectrometers (MS) were instrument of choice. Because of the different types of analyte and the determination of the different

analytes the MS was coupled to either an high performance liquid chromatography or to a gas chromatography. During this thesis, the following methods were developed for the quantitative detection of the following substances in different matrices:

- Propylene glycol, glycerol, nicotine in e-liquid, aerosol
- Propylene glycol and glycerol in various bodyfluids
- Nicotine, cotinine and nornicotine in plasma and saliva
- Carbonyles in aerosol and e-liquid
- Biomarkers of formaldehyde and acetaldehyde in plasma and urine
- Tobacco specific nitrosamines in aerosol

The methods mentioned above are described in detail in Section 4. They were validated according the FDA Guidelines for Bioanalytical Methods for Industry [179, 180].

2.5 Method validation

For preforming a method validation, the different authorities and council, such as the U.S. Food and Drug Administration (FDA)[180], European Medicines Agency (EMA)[181], Eurachem [182] and International Council for Harmonization (ICH)[183] have released guidelines for bioanalytical method validations. The main objective of method validation is to demonstrate the reliability of a particular method for the determination of an analyte concentration in the desired matrix.
The methods described in this work were validated according to the FDA Guideline Bioanalytical Methods for Industry 2013 and 2018, respectively [179, 180]. The guideline includes the following parameter limit of detection (LOD); limit of quantification (LLOQ); selectivity; calibration range; carryover; accuracy and precision; stability including short-term stability, freeze-thaw stability, post-preparative stability, long-term stability and stability of stock solutions; matrix effect; recovery and dilution effects. In the following section the interpretation of each parameter will be explained and the used acceptance criteria are pointed out.

Reference material

The reference material, if available, was purchased as certified reference material with an certificate of analysis. These certificate of analysis contained at least the source, the lot number, storage conditions, purity and the expiration date. If the standards were prepared internally, the identity was confirmed with tandem mass spectrometry and the purity by UV/VIS.
The already existing methods were additionally checked by interlaboratory tests, if they are available (e.g.: the methods for the mercapturic acids, nicotine and cotinine in urine and creatinine).

Limit of detection and limit of quantification

The limit of detection (LOD) describes the smallest possible value for a qualitative determination. It is accessed by determining the signal-to-noise ratio. This must be ≥ 3. The limit of quantification (LLOQ) is the theoretical value up to which a value can be quantified. This can also be determined from the signal-to-noise ratio (≥ 9), but it had to meet other criteria. This may result in the LLOQ of a method being higher than the theoretically determined value.

Acceptance criteria

- LLOQ signal had to be ≥ 5 times the signal from the S0.
- Accuracy and precision did not deviate more than ± 20 % from the nominal value. (Verification is performed as part of the determination of accuracy and precision (see Section Accuracy and Precision)).

Selectivity

Selectivity should demonstrate that the substance of interest and its internal standard can be quantified unhindered by matrix components. For methods with several analytes, it has to be eliminated that they influence each other during the measurement. For this purpose, 6 empty matrix samples (blanks), if possible from different sources, were analyzed. In addition, these 6 matrix samples were spiked in the lower calibration range and their accuracy, as well as their quantifier/qualifier ratio were determined. If no qualifier could be determined, the quantifier/qualifier ratio for the respective analyte was omitted. If no analyte-free matrix was available, only the accuracy of the spiking and the interference of the internal standard in the matrix sample were evaluated.

Acceptance criteria

- In the empty matrix samples there were no interference to the retention time of the analyte; the trace of the internal standard was not disturbed by more than 5 % of the mean value of the internal standard from the calibration.
- The Quan/Qual ratio may deviate ±25 % from the average Quan/Qual ratio from the calibration.
- The accuracy of the spiking may deviate ±15 % from the nominal spiked concentration.

Determination of the linearity range

The calibration curve had to include at least one blank (without analyte and internal standard; S00), one calibrator with internal standard only (S0) and 6 additional calibrators. The calibration curve covered the entire calibration range in each run, including the LLOQ. All blanks and calibrators were prepared in the same matrix as the expected study samples. If this is not possible, a surrogate matrix was used. This surrogate matrix had to have the same behavior as the matrix of the study samples.

Acceptance criteria

- The calibrators, except S00 and S0, did not deviate more than ± 15 % from the nominal concentration with the exception of the calibrator at LLOQ; this may deviate by ± 20 %.
- 75 % of the calibrators and at least 6 calibrators had to meet the above criteria in each run.

Carryover

Carryover can occur when the concentration of a sample is high. This carryover then affects the result of the subsequent measurement. To determine carryover, the highest calibrator was injected 5 times, followed by a blank sample. Then the highest calibrator was injected 5 times again, followed by a blank sample. The procedure was repeated once more. If the carryover was large than 20 % of the area of the lowest calibrator, the calibration range must be adjusted accordingly.

Acceptance criteria

- The area of the signal in the blank had to be less than 20 % of the area of the lowest calibrator.

Accuracy and precision

The accuracy and precision of a method were determined over the entire calibration range. Accuracy is a measure of the correlation between the measured value (x_i) and the reference value (x_{Ref}). Precision, on the other hand, indicates the variation of the measured values among each other ($\bar{x}$).

$$\text{Accuracy} = \frac{|x_i - x_{Ref}|}{x_i} \tag{4}$$

$$\text{Standard deviation (s)} = \sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2} \tag{5}$$

$$\text{Variation coefficient (v)} = \frac{s}{\bar{x}} \tag{6}$$

Verification of accuracy and precision was performed on 3 different days (interday) and 5 replicates per day (intraday). For this purpose, blank samples were spiked with 4 different concentrations of the analyte (at the LLOQ, in the lower (L), middle (M) and upper (H) calibration range). This was done on a daily basis and for each sample individually. The measurement results were evaluated using these formulas (4), (5) and (6) and then evaluated using the following acceptance criteria.

Acceptance criteria

- Accuracy: The deviation from the nominal value did not exceed ± 15 %, except at LLOQ where ± 20 % applies.
- Precision: The coefficient of variation did not deviate by more than ± 15 %, except at LLOQ, where ± 20 % applies.

Stability

In the following, 5 different criteria are explained which were used to check the stability of the analyte in the matrix, in the stock solution, and in the processed sample. Different temperature ranges and storage periods were considered. The selection of the criteria depends on the method. However, short-term stability and freeze-thaw stability were performed in any case.

Short-term stability: For the determination of the short-term stability, 3 low-spiked and 3 high-spiked samples were left overnight ($>$ 16 h) at room temperature. The next day, these samples were processed with a fresh calibration and the nominal concentration were compared to the measured concentration.

Freeze-thaw stability: To determine the freeze-thaw stability, 3 low-spiked and 3 high-spiked samples were freshly prepared and measured. The samples were frozen overnight ($>$ 16 h) and thawed during the day. This is one cycle. 6 cycles should be performed. If fewer freeze-thaw cycles were required within the method, this was justifiably adjusted. Sample concentrations were measured and compared with a fresh calibration.

Post-preparative stability: Post-preparative stability reflects the stability of the sample after processing and storage. 6 processed samples (3 low-spiked and 3 high-spiked each) remained in the autosampler or freezer. The maximum time of stay was evaluated in this experiment. The re-injected samples were analyzed with a fresh calibration and compared to the initial value.

Long-term stability: Long term stability covers the time period from initial sample collection to analysis. 3 low-spiked and 3 high-spiked samples were stored at the expected storage temperature and tested against a fresh calibration at the expected time interval.

Stability of stock solutions: The stock solution was prepared fresh from reference material. The fresh stock solution and the stock solution to be tested were diluted into the calibration range and compared.

Acceptance criteria for the above described parameters

- Accuracy: The deviation from the nominal value must not exceed ± 15
- Precision: The coefficient of variation must not deviate by more than ± 15 %.

Matrix effect

The matrix may have various influences on the ionization of the analyte (ion suppression or increased ionization). These influences should be compensated by the choice of a suitable internal standard. To demonstrate this compensation, both the internal standard and the analyte are spiked at low and high concentrations into a matrix sample and into a reference solution. The signal areas obtained from the matrix sample and the reference solution are compared and a recovery is calculated. A suitable internal standard should be within ± 15 % of the analyte recovery.
If an analyte-free matrix was not available, an infusion experiment was performed. In an infusion experiment, the reference solution and the matrix sample are measured consecutively. During these measurements, the analyte or internal standard is permanently infused into the MS. For this purpose, the analyte and the internal standard (in 2 different concentrations) are diluted in a reference solvent and infused directly into the LC flow after the chromatographic separation. The signal intensity of the analyte or internal standard from the reference solution is compared to those from the matrix sample. The increase or decrease, respectively, of the signal was used to determine the matrix effect. This deviation between the analyte and its internal standard should not be more than 15 %.

Recovery

Recovery was determined if an extraction step was included in the sample preparation. 3 samples without internal standard at each level (low, medium high) were processed. In addition, 9 blank samples are processed and after extraction the were spiked with the analyte at the same levels (low, medium, high). Internal standard is added to all samples immediately prior to measurement.

Dilution effects

The dilution effects were determined if dilution was planned as part of the sample preparation. For this purpose, 3 different solutions were prepared with different concentrations above the calibration range. These 3 solutions are diluted in 3 different dilution steps (which are also necessary within the method). Each dilution step must be measured in a 5-fold determination.

Acceptance criteria

- Accuracy: The deviation from the nominal value did not exceed $\pm 15\,\%$, except at LLOQ where $\pm 20\,\%$ applies.
- Precision: The coefficient of variation did not deviate by more than $\pm 15\,\%$, except at LLOQ, where $\pm 20\,\%$ applies.

Quality Controls

The quality controls (QCs) were prepared in the same matrix as the study samples. Where possible, QCs were prepared from authentic material. For this reason, matrix samples with similar concentration were pooled to get 3 different concentration which covered the whole calibration range. If there were no matrix samples in 3 different concentrations, the matrix samples were either diluted or additionally spiked with the analytes to the desired concentration.
The QCs should document the accuracy and precision of a method throughout the measurement. Within a measurement, there were at least 6 QCs (low (L), medium (M), and high (H) concentration range) with 3 different concentrations covering the entire calibration range. For larger numbers of study samples, 5 % of the study samples were QCs.

Acceptance criteria

- $\geq 67\,\%$ of the QCs deviate less than $\pm 15\,\%$ from the theoretical value (unless the concentration of the QC sample was at the LLOQ, in which case the deviation did not exceed $\pm\, 20\,\%$).
- $\geq 50\,\%$ of a QC level deviate less than $\pm 15\,\%$ from the theoretical value (unless the concentration of the QC sample was at the LLOQ, in which case the deviation did not exceed $\pm\, 20\,\%$)

3 Research Aims

The present research project should provide answers to the following two primary questions:

- What are the pharmacokinetics and the uptake dose of the major e-liquid constituents PG, G and N in vapers under controlled and free vaping conditions in relation to the electrical power (wattage) of the e-cigarette used?
- Do vapers take up toxicants such as formaldehyde, acetaldehyde, acrolein, ethylene oxide, propylene oxide, glycidol, crotonaldehyde, NNN and NNK, which, at least theoretically, can be formed from the precursors PG, G and N in e-liquid?

In addition, the following secondary questions should be answered:

- How do pharmacokinetics and exposure doses compare to smokers of conventional cigarettes (CC)?
- Which body fluids (blood, saliva, urine) are suitable for measuring the vaping-related biomarkers?
- Can the major e-liquid constituents PG, G and N be found in sputum samples of vapers, and if so, how long are they retained in the respiratory tract?
- Can the (theoretically) assumed toxicants formaldehyde, acetaldehyde, acrolein, ethylene oxide, propylene oxide, glycidol, crotonaldehyde, NNN and NNK be detected in the e-cigarette aerosol, when generated by a smoking machine under high and low wattage conditions and, if so, what are the yields?

4 Experimental

4.1 Clinical Study

4.1.1 E-Cigarette, e-liquid and cigarette

The e-cigarette is a tank-based e-cig which consists of the rechargeable battery vaporizer Eleaf iStick TC 40W (Irvine CA, USA) and the tank system Aspire Nautilus mini 2 ml 1.8 Ω (Kent, WA, USA). The vaporizer has a power range from 1 to 40 W. The tank system consists of a liquid glass tank and a replaceable vaporizer. The liquid tank has a capacity of 2 ml. The replaceable atomizer has a resistor of 1.8 Ω. All parts are manufactured in agreement with EU legislation and they are CE labeled.
Custom-made e-liquids were supplied by the company 'Happy Liquid' (Munich, Germany). The targeted composition of the e-liquid was PG/G (50:50, w/w), 12 mg/ml nicotine and an American blend tobacco aroma. All materials used were EP grade. For use in the clinical study, 10 % of the e-liquid were replaced with a mixture of $^{13}C_3$-PG, $^{13}C_3$-G and nicotine-d_7 in the same proportions as the unlabeled e-liquid. Labeled reference standards $^{13}C_3$-PG (isotopic purity: 97 %), $^{13}C_3$-G (isotopic purity: 99 %) and nicotine-d_7 (isotopic purity: 97 %) were purchased from Synthèse AptoChem Inc. (Montreal Canada). Certificates of analysis (CoAs) for the stable-isotope labeled compounds comprise water content, residual solvents, identity, chromatographic purity, "as is" potency, lot number, manufacturing date, and expiration date. The CoAs also contain a statement that these compounds can be used in e-cigarettes as part of a clinical study. Prior to use in the study, the final e-liquid was analyzed for the unlabeled and labeled major components (described in Section 4.2).
As a 'positive control' (in terms of pyrolytic formation of toxicants for the labeled precursors PG, G and nicotine), non-filter cigarettes from the German market (ISO yields: 10 mg tar, 0.32 mg nicotine, 10 mg carbon monoxide) were spiked with 13.4 mg $^{13}C_3$-PG, 13.6 mg $^{13}C_3$-G, and 2.4 mg nicotine-d_7 dissolved in 100 µl ethanol. The spiking solution was evenly distributed along the central axis of the tobacco rod, using a needle-armed syringe. Subsequently, the spiked CCs are dried at room temperature for 24 hours in order to evaporate the residual ethanol.

4.1.2 Study design

A clinical study was conducted at the Clinical Trial Center (CTC) North (Hamburg, Germany). Ethical approval was received according to the Helsinki declaration [184] by the Ethical Commission of the Medical Chamber of Hamburg (Germany). The study included 25 healthy male Caucasian volunteers aged 21 to 60 years and a body mass index (BMI) between 18 and 30 kg/m^2. Five regular current smokers of $\geq$ 10 cigarettes/d and 20 experienced vapers with a regular consumption of $\geq$ 1.5 ml of nicotine containing e-liquid per day and without use of other nicotine products (cigarettes, chewing gum, patch, inhaler) were recruited for the study. The clinical study was divided into two study parts (Part A = stationary phase and Part B = ambulatory phase). In Part A, the subjects stayed for 84 h in the clinic (from afternoon of Day -1 until the morning of Day 4). After the subjects arrived in the clinic they were not allowed to smoke or vape until Day 1 of the study. On Day 1, half of the vapers (N=10, randomly assigned) consumed the provided labeled e-liquid (described above) at high wattage conditions (18 W, 18 W vaper) during 10 defined vaping sessions, each comprising

10 puffs at intervals of 0.5 minutes. The other 10 vapers consumed the e-liquid under the same conditions apart from the wattage, which was set to 10 W (low wattage conditions, 10 W vaper). Wattage conditions are selected in a manner that resembles popular, usual and convenient e-cigarette vaping conditions of this e-cigarette type. The 5 smoker consumed 10 non-filter cigarettes spiked with ^{13}C-labeled PG and G as well as deuterated nicotine (nicotine-d_7) as a 'positive control'. Controlled vaping and smoking session took place at 9, 10, 11, 12 a.m. and at 2, 3, 4, 5, 6 p.m. on Day 1. After the 10 sessions, the subjects were prohibited to vape or smoke for the remaining Part A (evening of Day 1 until morning of Day 4). There was a wash out period of at least seven days between the Part A and B. In the ambulatory Part B, only the 20 vapers (the same subjects which participated in Part A) were provided with the same type of e-cigarette as used in Part A (including the stable-isotope labeled e-liquid). Subjects were advised to vape ad libitum during the duration of Part B of the study (72 h) with the provided product only. Actual vaping in terms of time of the day, number of puffs taken and wattage adjustment was recorded in protocol forms (see Appendix B) by the subjects. Subjects came to the clinic on each evening of the 3 study days as well as on the evening of Day -1 (start of Part B). The time lines of the entire study per subject are visualized in Figure 9.

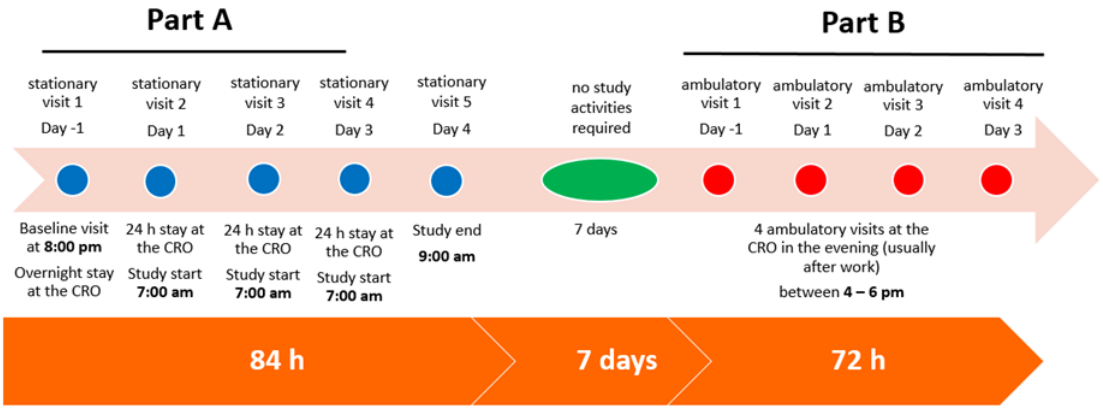


Figure 9: Outline of the study design

The stationary Part A was conducted under strictly controlled dietary conditions to minimize nutritional effects. During the stationary visits, three meals were served during the day; breakfast at 9 a.m., lunch at 12 a.m., and dinner at 7 p.m. The caloric intake was normalized to the weight of the subjects in five groups according to the Harris-Benedict equation [185]. Therefore, the amount of lunch was adjusted, while all subjects were served the same breakfast and dinner. The nutrition composition was served as defined prior to the study, comprising 72 % carbohydrates, 14 % proteins, and 14 % fat. For standardization purposes the subjects were asked to consume the complete quantity served. Deviations concerning the food uptake were documented. Subjects were only allowed to drink water without any restriction concerning the amount. During the ambulatory Part B, the subjects were not restricted in terms of food and drinks.

4.1.3 Sampling

Urine, plasma, saliva and sputum samples were collected for the determination of biomarkers in both parts of the clinical study at the time points shown in Figure 10. In Part A, baseline urine samples (U0, spot urine) were collected at 8 a.m. Subjects were advised to completely collect all urine voids between 9 a.m. and 9 p.m., preferably with intervals of 1-2 h in separate bottles on Days 1, 2 and 3. The last urine sample was collected in the morning of Day 4. The individual urine voids were divided into 6 intervals (U1-Ux) between 9 a.m. and 9 p.m. each two hours long. Exact collection times and masses were recorded. Blood samples were drawn immediately before and after the 10 vaping/smoking sessions as well as 1 and 2 h after the last session on Day 1. On Day 2 and 3 of Part A, the samples were drawn only three times per day, at 8 a.m., 1 p.m. and 9 p.m.. The last blood sample was collected in the morning of Day 4. All blood samples were drawn in EDTA monovettes (4.9 ml, Sarstedt, Nuembrecht, Germany) and centrifuged within 1 h. Saliva samples of about 2 ml were collected at virtually the same time points as the blood samples in Part A according to the method of Navazesh [186]. Subjects were not allowed to eat, drink (except water) or use any oral care products 20 min prior to saliva sampling. Immediately before the collection, subjects rinsed their oral cavity three times with water. Non-induced sputum samples were collected according a clinical routine procedure [187] at about 8 a.m. and 9 p.m. on Day 1, 2 and 3 as well as in the morning of Day 4 of Part A of the clinical study under supervision of the medical staff. All obtained samples were immediately stored at -20 °C until analysis.

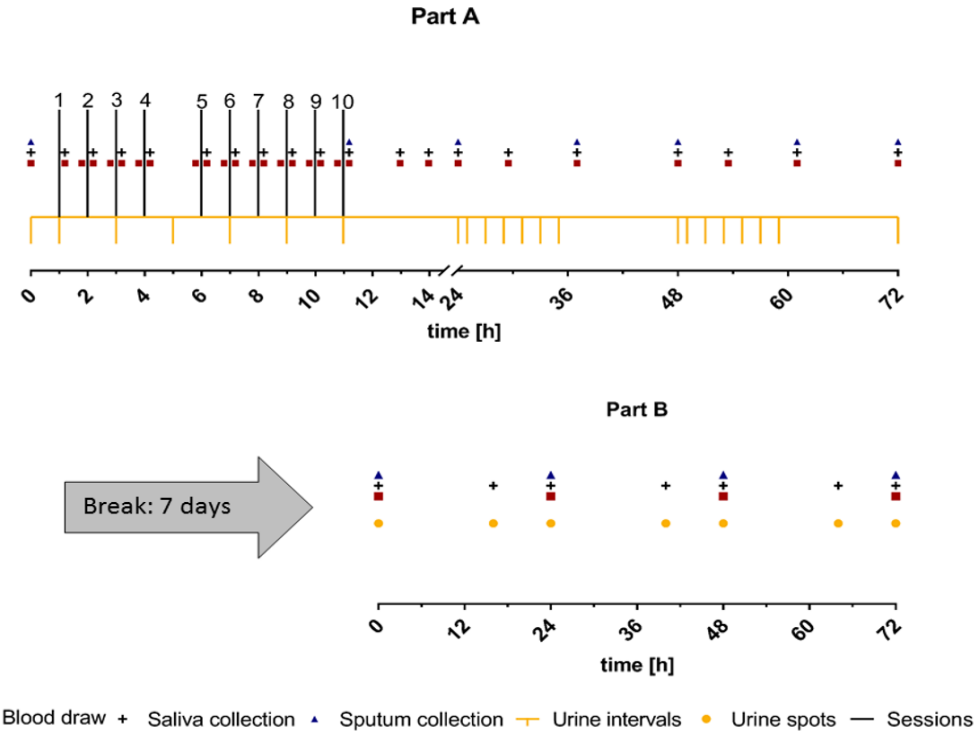


Figure 10: Time scheme for sampling of the clinical study. Lines 1 – 10 indicate time points for the vaping/smoking session. Sample collection is marked with various symbols.

In Part B of the clinical study, subjects provided spot urine samples at their ambulatory visits in the evening of study Days -1, 1, 2 and 3. The morning urine samples of Days 1, 2 and 3 were collected at home, stored in a cooling bag at 4 – 6 °C until the samples were brought to the clinic in the evening. Saliva samples in Part B were collected at about similar time points as the spot urine samples according to the procedure described for Part A. Blood samples in Part B were drawn at the occasion of the ambulatory visits to the clinic on Days -1, 1, 2 and 3. Sputum samples were collected under supervision in the evenings of Days -1 and 3 of Part B. All sampling times were recorded and samples were stored as described for Part A.

4.2 Liquid and aerosol characterization

For the analysis of the aerosol and smoke, respectively, a combined setup was used. This setup is schematically shown in Figure 11. It includes Cambridge filters (44 mm, Borgwaldt, Hamburg, Germany), two glass impingers and a 1-channel smoking machine (RM1, Borgwaldt, Hamburg, Germany). The analytes were trapped either on the Cambridge filter or in the glass impinger depending on their properties. The aerosol/smoke was generated by applying 55-ml puff volume, 4-s puff duration and a 30-s puff interval according to the vaping/smoking patterns during the clinical study. The amount of the trapped puffs vary between the different methods due to the detection limits of the analytes. The e-cigarette tank with the e-liquid is weighted before and after the trapping procedure. The amount of consumed e-liquid, besides the numbers of puff, is another way to normalize the data.

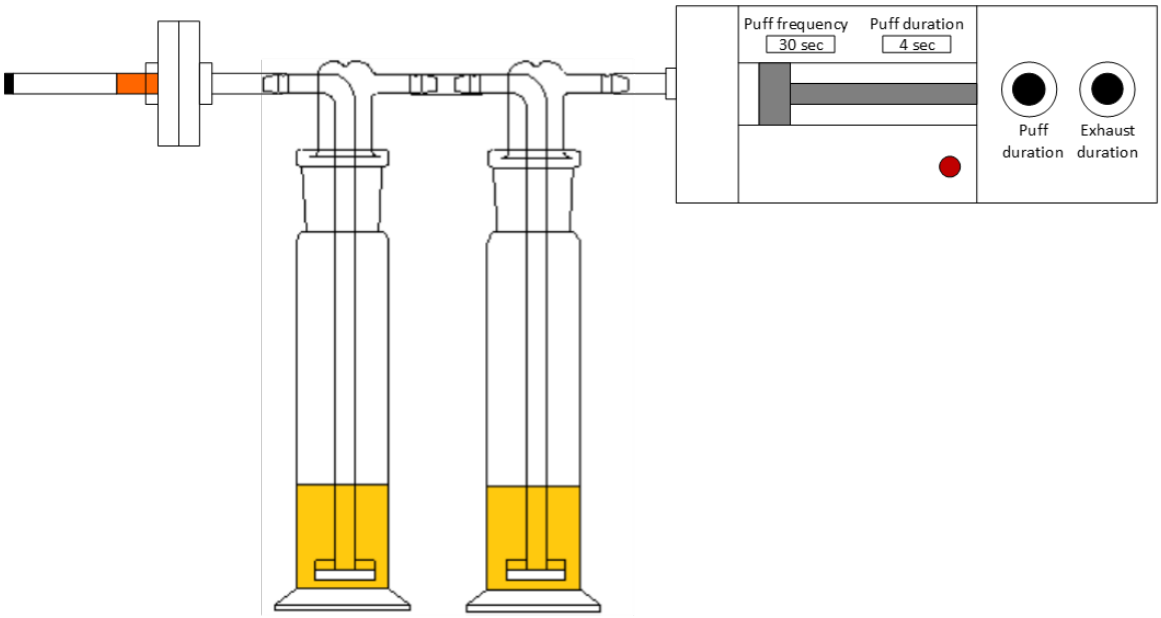


Figure 11: Scheme of the aerosol/smoke trapping. Showing the filter holder with a Cambridge filter, two glass impingers with derivatization agent and the smoking machine with the adjustable parameters

All the described methods were developed, validated and established during this project and will be presented in the following sections.

4.2.1 Propylene glycol, Glycerol and Nicotine

4.2.1.1 Chemicals and solutions

Glycerol ($\geq$ 99 %), nicotine ($\geq$ 99 %), propylene glycol ($\geq$ 99.5 %) and butane-1,2,4-triol ($>$ 97 %) were purchased from Sigma (Taufkirchen, Germany). d_6-propylene glycol ($\geq$ 98 %) and d_3-nicotine ($\geq$ 96 %) were obtained from CDN Isotopes Inc. (Quebec, Canada). $^{13}C_3$-Glycerol (99 %), $^{13}C_3$-propylene glycol ($\geq$ 96 %) and nicotine-d_7 ($\geq$ 98 %) were purchased from Synthèse AptoChem (Montréal, Canada). Ethanol (Optigrade) was obtained from Promochem (Wesel, Germany). Ultrapure water was prepared by arium® pro ultrapure water system (Sartorius, Göttingen, Germany). The Cambridge filter (44 mm) were purchased from Borgwaldt (Hamburg, Germany).

A standard stock solution (SL) of PG and G (10 mg/ml) in ethanol and their ^{13}C-labeled analogues (1 mg/ml) in DI water as well as the SL of nicotine-d_7 (1 mg/ml) were prepared. Nicotine was purchased as a SL in methanol (1 mg/ml). The calibration solution was prepared by adding 500 µl of PG-SL and G-SL, 1 ml $^{13}C_3$-PG-SL and $^{13}C_3$-G-SL, 150 µl of N-SL and 50 µl N-d_7 to a 5-ml flask and diluting to the mark with ethanol. This calibration was achieved by diluting the calibration solution 1:2 until they reached the concentration 30 µg/ml PG/G and 0.9 µg/ml N or 3 µg/ml $^{13}C_3$-PG/$^{13}C_3$-G and 0.09 µg/ml N-d_7, respectively. Due to some interference of the deuterated G, butane-1,2,4-triol was used as internal standard for G. PG-d_6 and N-d_3 were combined with the butane-1,2,4-triol to the IS-Mix (PG-d_6: 1 mg/ml; N-d_3: 0.05 mg/ml; butane-1,2,4-triol: 0.5 mg/ml).

4.2.1.2 Instrument and software

The GC-MS system is a combination of the GC 6890N (Agilent, Waldbronn, Germany) with the Cooled Injection System CIS 4 (Gerstel, Mühlheim an der Ruhr, Germany), the autosampler MPS 2 (Gerstel, Mühlheim an der Ruhr, Germany) and the detector MSD 5975 (Agilent, Waldbronn, Germany).

For the generation and the processing of the data Agilent ChemStation 2.0 (Agilent Technologies, Santa Clara, USA) and MassHunter Workstation Software B.07.00 (Agilent, Santa Clara, USA) were used.

4.2.1.3 Sample preparation and analytical methodology

The labeled e-liquid mixture (prepared as described in Section 4.1.1) was analyzed for its content of labeled and unlabeled PG, G as well as nicotine by means of a GC-MS method. The e-liquid was diluted 1:1000 with ethanol and 20 µl IS-Mix was added to 200 µl of the diluted sample. One µl was injected into a GC-MS system operated in the electron ionization mode at 70 eV with a filament delay of 2.4 min. The analytes were quantified in the selected ion monitoring (SIM) mode using mass fragments m/z 61 (PG/G), 63 ($^{13}C_3$-PG/$^{13}C_3$-G), 133 (N) and 139 (N-d_7), as well as 57 and 136 for the IS butane-1,2,4-triol, and d_3-nicotine, respectively. Analytes were separated on a mid polarity cross bond phase RTX-1701 column (30 m x 0.25 mm i.d. 0.25 µm df, Restek, Bad Homburg, Germany) using helium as carrier gas at a constant flow of 1.5 ml/min. The split/splitless injector was set to 230 °C and operated at a split of 1/2. The temperature program of the GC oven was used as follows: the initial oven temperature was set to 65 °C for 1 min, then it was raised to 185 °C with a rate of 40 °C per min. Subsequently the temperature was raised to 240 °C with a rate of 20 °C per min and it was held for 7.75 min.

The aerosol/smoke was trapped on a Cambridge filter using the puff regime described in Section 4.2. 20 puffs of the e-cigarette at both wattage conditions (10 and 18 W) as well as a single cigarette were trapped on separate 44-mm Cambridge filters (Borgwaldt, Hamburg, Germany). The filters were extracted for 40 min with 8 ml ethanol and afterwards they were diluted 1:10. The diluted extracts were analyzed for labeled and unlabeled PG, G as well as nicotine with the GC-MS method described above.

4.2.1.4 Validation

The analytical work flow was validated according to the FDA Guideline 2018 [180] with slight modi-fications due to the matrix and sampling procedure. The validation does not cover the trapping efficiency during the sample collection. Only the carryover to a second filter or glass impinger was tested with the result that one filter is sufficient for trapping. The three analytes were not detected on the second filter and the first impinger. During this work, it was investigated that 50 % of the e-liquid is lost in the (e.g.: through condensation in the tubing, see Section 6.1).

The following validation criteria were investigated for the trapped analytes: selectivity, linearity, range, accuracy, precision, recovery, matrix effect and stability.

The specificity of the method was ensured by the fact that no interfering signals at the expected RT in extracted analyte-free filters were detected for the quantifier mass.

Intra- and interday precision along with the accuracies of extracts of spiked filters were tested at 4 different concentration levels at 3 different days. The precisions across all analytes were better than 9 % (at LLOQ: 14 %). The accuracies of the high and medium levels were within the range 95 - 114 % and for the low levels between 83 % and 118 %.

The LODs and LLOQs were defined as S/N ratios 3:1 and 9:1, respectively. The LODs were calculated by the software using the signal-to-noise algorithm (PG: 0.4 µg/ml, G: 3.4 µg/ml and N: 0.2 µg/ml) The LLOQs are more than a factor 3 higher than the LODs due to the background contamination from the environment and the used material. The LLOQ was set to 30 µg/ml for PG and G and 0.9 µg/ml for N.

The recovery rate was for all analytes between 93 % and 106 % by comparing the extract from the filter and an ethanol solution with the expected concentration. In both matrices (aerosol/smoke), matrix effect was not detected.

The calibration ranged from 0.03 mg/ml to 1 mg/ml for PG/G and from 0.9 - 30 µg/ml for N which covers the expected amounts of the analytes in the e-liquids. No carryover was observed from the highest calibrator to a blank sample.

The stability was characterized by means of the short-term and post-preparative stability as well as one freeze-thaw cycle. All analytes were found to be stable for 16 h at room temperature. The post-preparative stability was even proven for 16 days in the autosampler (15 °C). The freeze-thaw cycle was tested with the non-extracted filter for one cycle. It was found that all analytes are stable.

The detailed validation protocol is summarized in Appendix A Table 17.

4.2.2 Carbonyls

4.2.2.1 Chemicals

Acetaldehyde-DNPH (99.9 %), acetone-DNPH (99.7 %) acrolein-DNPH (99.8 %), formaldehyde-DNPH (99.9 %), crotonaldehyde-DNPH (99.6 %), methacrolein-DNPH (96.7 %) and propionaldehyde-DNPH (98.3 %) were purchased from Neochema (Bodenheim, Germany). 3,5,6-d_3-acetaldehyde-DNPH (99 %), 3,5,6-d_3-acetone-DNPH (99 %), 3,5,6-d_3-acrolein-DNPH (99 %), 3,5,6-d_3-formaldehyde-DNPH (98.8 %), 3,5,6-d_3-crotonaldehyde-DNPH (99 %) and 3,5,6-d_3-propionaldehyde-DNPH (98.6 %) were obtained from CDN Isotopes Inc. (Quebec, Canada). 2,4-Dinitrophenylhydrazine (DNPH)

for HPLC derivatization (> 99 %), perchloric acid (70 %) and pyridine (anhydrous, 99.8 %) were purchased from Sigma (Taufkirchen, Germany). Acetonitrile (ULC/MS grade) was obtained from Biosolve BV (Valkenswaad, Netherlands). Ultrapure water was prepared by arium® pro ultrapure water system (Sartorius, Göttingen, Germany). The concentration of the standards and the internal standards are based on the calculated concentration of the free aldehyde (see formula 7).

$$\text{c (free aldehyde)} = \text{c (aldehyde-DNPH-derivate)} * \frac{\text{M (free aldehyde)}}{\text{M (aldehyde-DNPH-derivate)}} \quad (7)$$

c = concentration [g/l]
M = molar mass [g/mol]

The standards were diluted to two different working solutions AL I and AL II in acetonitrile with a concentration of 500 ng/ml and 10 ng/ml, respectively. The deuterated internal standards were desolved in acetonitrile and each diluted to 500 ng/ml (IS-Mix).
An acidic 2.5 mM DNPH solution was prepared by solving 124 mg DNPH in 250 ml acetonitrile. 40 µl perchloric acid were added to this solution.

4.2.2.2 Instrument and software

A Shimadzu Nexera X2 UPLC system including a binary pump, an autosampler, a degasser and a column oven (Shimadzu Corp., Kyoto, Japan) and a triple quadrupole mass spectrometer QTRAP® 6500^+ equipped with a Turbo V ion spray source, operating in negative ESI mode, was used for detection (AB Sciex, Darmstadt, Germany). High purity nitrogen (purity: 99.7 %) was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).
For the generation and the processing of the data Analyst® 1.6.3 (Sciex, Darmstadt, Germany) was used.

4.2.2.3 Sample preparation and analytical methodology

Since the acetaldehyde, acetone, acrolein, crotonaldehyde, formaldehyde, methacrolein and propionaldehyde are highly volatile and reactive, they are derivatized upon trapping, followed by dilution. According to Miller et al. [188], 10 puffs of the e-cigarette or a single cigarette (puffing regime according to section 4.2) were drawn through two glass impingers which both contained 20 ml of an acidic 2.5 mM DNPH derivatization solution. After the trapping experiment, 200 µl pyridine were added to stop the derivatization. The trapped derivatives were diluted 1:10 (1:100 for the cigarette). Prior to the UPLC-MS/MS analysis, 10 µl IS-Mix were added to 100 µl of the diluted sample. For the determination of the background contamination an aliquot was drawn on a daily basis and analyzed with the samples.
Additionally the e-liquid was tested for carbonyl formation during storage. 100 µl e-liquid were diluted with 900 µl acidic 2.5 mM DNPH solution and agitated for 5 min. 10 µl pyridine were added to stop the derivatization and 100 µl sample were transferred into a microvial containing 10 µl IS-Mix.
5 µl of the sample were injected on a Kinetex® 5 µm EVO C18 column (150 x 2.1 mm, 5 µm, Phenomenex, Aschaffenburg, Germany). The chromatographic separation was achieved with water

(eluent A) and acetonitrile (eluent B) following this gradient elution: : 0-5.0 min: 35-55 % B; 5.0-7.0 min: 70 % B; 7.0-7.1 min: 70-35 % B; 7.1 – 10.0 min: 35 % B. The column was kept at 50 °C with a flow rate of 0.7 ml/min. The turbo-ion spray source settings were as follows: ion spray voltage = -4000 V, heater temperature = 600 °C, source gas 1 = 4.8 bar, source gas 2 = 5.5 bar, CAD gas = medium and curtain gas = 2.8 bar. Labeled and unlabeled analytes as well as their corresponding internal standards were monitored in the multiple reaction monitoring mode (MRM, Table 3) with a MRM detection window of 60 sec using negative electrospray ionization (ESI^-).

Table 3: MS/MS-parameters for the labeled and unlabeled carbonyl-DNPH-derivates and their corresponding internal standards sorted by retention time (RT). (IS: internal standard, MRM: multiple reaction monitoring)

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	IS	IS MRM [m/z → m/z]
Formaldehyde	2.1	209 → 163	209 → 151	d_3-Formaldehyde	212 → 166
^{13}C-Formaldehyde		210 → 164	210 → 151		
Acetaldehyde	2.8	223 → 163	223 → 151	d_3-Acetaldehyde	226→ 166
$^{13}C_2$-Acetaldehyde		225 → 164	225 → 151		
Acetone	3.6	237 → 151	237 → 207	d_3-Acetone	240→ 154
$^{13}C_3$-Acetone		240 → 151	240 → 210		
Acrolein	3.7	235 → 163	235 → 158	d_3-Acrolein	238→ 166
$^{13}C_3$-Acrolein		238 → 164	238 → 158		
Propionaldehyde	4.0	237 → 163	237 → 179	d_3-Propionaldehyde	240→ 166
$^{13}C_3$-Propionaldehyde		240 → 164	240 → 180		
Crotonaldehyde	4.6	249 → 172	249→ 151	d_3-Crotonaldehyde	252→ 175
$^{13}C_2$-Crotonaldehyde		251 → 173	251→ 151		
$^{13}C_4$-Crotonaldehyde		253 → 173	253→ 151		
Methacrolein	5.0	249 → 163	249 → 122		
$^{13}C_2$-Methacrolein		251 → 164	251 → 122		
$^{13}C_4$-Methacrolein		253 → 164	253 → 122		

4.2.2.4 Validation

The analytical work flow was validated according to the FDA Guideline 2018 [180] with slight modi-fications due to the matrix and sampling procedure. The validation does not cover the trapping efficiency during the sample collection. Only the carryover to a second glass impinger was tested with the result that one impinger is sufficient for trapping. The measured concentration of the carbonyles in the second impinger were as large as the daily determined background concentration in the DNPH-solution. The following validation criteria were investigated: selectivity, linearity, range, accuracy, precision, matrix effect and stability.

The specificity of the method was ensured by the fact that no interfering signals at the expected RT

of the internal standard were found and that the spiked amounts were found in the matrix within an accuracy of 90 and 115 %.
Intra- and interday precision along with the accuracies were tested at 4 different concentration levels at 3 different days in the undiluted and diluted (1:10, 1:100) DNPH solution . The precision across all analytes were better than 13 %. The accuracy of the high and medium levels were within the range 90 - 115 % and for the low levels between 80 % and 120 %.
The LODs and LLOQs were defined as S/N ratios 3:1 and 9:1, respectively. The LODs were calculated by the software using the signal-to-noise algorithm (LOD: 0.03 ng/ml). The LLOQ was set to 0.1 ng/ml for all analytes. The calibration ranged from 0.1 ng/ml to 500 ng/ml. No carryover was observed from the highest calibrator to a blank sample.
Accuracy and precision after dilution were determined in the two different matrices (smoke and aerosol) at three different levels. The accuracy was within 86 and 111 % and the coefficient of variation was lower than 10 %.
The stability was characterized by means of the short-term and post-preparative stability as well as freeze-thaw cycles. All analytes were found to be stable for 16 h at room temperature. The post-preparative stability was even proven for 10 days in the autosampler (10 °C) except for formaldehyde. Formaldehyde is enriched as soon as the vials are punctured, so there is no post-preparative stability. If the sample must be measured repeatedly, a new dilution must be made from the original sample. In addition, the blank must also be determined again. All analytes are stable for 3 freeze-thaw cycles with the exception of acetone. In the undiluted solution, acetone is stable for only one freeze-thaw cycle. Therefore, a 1:10 diluted aliquot must be prepared in sufficient quantity before the first freeze-thaw cycle. The stability of the stock solutions was checked for formaldehyde, acetaldehyde, crotonaldehyde and acrolein. The stock solution for the other carbonyls has not yet been checked. To check the stability of the stock solutions, stock solutions from 2016 and current stock solutions (2019) were identically diluted and compared. The stock solutions are stable for 2.5 years.
The matrix effect was determined in different matrices (cigarette, e-cigarette and DNPH-solution) in 2 different concentrations. 100 µl of the solution was spiked with 10 µl standard solution. As reference 100 µl ACN were spiked with 10 µl reference solution. No matrix effect was observed.
The detailed validation protocol is summarized in Appendix A, Table 18 - 20.

4.2.3 Epoxides

Various trapping agents and chromatographic conditions were tested for the determination of the epoxides ethylene oxides (EO), propylene oxide (PO) and glycidol (GLY) in e-cig aerosol and mainstream smoke. The analytical method showing the best performance with regard to the sensitivity and recovery used the trapping agent toluene and the polar GC column a PoraBOND Q (25 m x 0.25 mm, 0.25 µm; Agilent, Waldbronn, Germany). However, the sensitivity of the final method with an LLOQ of 0.05 µg/ml was still not sufficient for the quantification of EO, PO and GLY in this project.

4.2.4 Tobacco specific nitrosamines

4.2.4.1 Chemicals

N-Nitrosoanabasin (NAB, 98 %), N-Nitrosoanatabin (NAT, 98 %), N-Nitrosonornicotin (NNN, 98 %), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, 98 %) and NNK-d_3 (98 %) were purchased from Toronto Research Chemicals Inc. (North York, Canada). Ammonium acetate (UPL/MS grade) and acetonitrile with 0.1 % formic acid (UPL/MS grade) was obtained from Biosolve BV (Valkenswaad, Netherlands). Ultrapure water was prepared by arium® pro ultrapure water system (Sartorius, Göttingen, Germany). The Cambridge filter (44 mm) were purchased from Borgwaldt (Hamburg, Germany).
The IS-Mix contains only NNK-d_3 (12.5 µg/ml) because the corresponding deuterated internal standards of NAB, NAT and NNN are potentially occurring decomposition products of nicotine-d_7.
3.9 g of ammonium acetate were dissolved in 500 ml ultrapure water yielding a 100 mM ammonium acetate buffer.

4.2.4.2 Instrument and software

A Shimadzu Nexera X2 UPLC system including a binary pump, an autosampler, a degasser and a column oven (Shimadzu Corp., Kyoto, Japan) and a triple quadrupole mass spectrometer QTRAP® 6500^+ equipped with a Turbo V ion spray source, operating in positive ESI mode, was used for detection (AB Sciex, Darmstadt, Germany). High purity nitrogen (purity: 99.7 %) was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).
For the generation and the processing of the data Analyst® 1.6.3 (Sciex, Darmstadt, Germany) was used.

4.2.4.3 Sample preparation and analytical methodology

Prior to the trapping, 10 µl IS-Mix were spiked on the Cambridge filter. 20 puffs of the e-cigarette or a single cigarette were trapped on a filter (puff regime described in Section 4.2). The filter was extracted with 10 ml 100 mM ammonium acetate buffer by rolling the sample for 40 min. 100 µl are transferred into a microvial.
The e-liquid was analyzed by dilution 100 µl e-liquid with 900 µl 100 mM ammonium acetate buffer. 10 µl IS-Mix are added to 100 µl of the diluted e-liquid.
10 µl of the sample were injected on a Kinetex® 5 µm EVO C18 column (150 x 2.1 mm, 5 µm, Phenomenex, Aschaffenburg, Germany). The chromatographic separation was achieved with 0.1 % ammonium acetate buffer (eluent A) and acetonitrile with 0.1 % formic acid (eluent B) following this gradient elution: : 0-0.5 min: 10 % B; 0.5-2.0 min: 10 - 75 % B; 2.0-3.5 min: 75 % B; 3.5 – 6.0 min: 10 % B. The column was kept at 50 °C with a flow rate of 0.45 ml/min. The turbo-ion spray source settings were as follows: ion spray voltage = 5500 V, heater temperature = 500 °C, source gas 1 = 4.8 bar, source gas 2 = 5.5 bar, CAD gas = medium and curtain gas = 3.4 bar. Labeled and unlabeled analytes as well as the internal standard were monitored in the MRM mode (Table 4) using positive electrospray ionization (ESI^+) .

Table 4: Mass transition and retention time (RT) of labeled and unlabeled N-Nitrosoanabasin (NAB), N-Nitrosoanatabin (NAT), N-Nitrosonornicotin (NNN), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). MRM: multiple reaction monitoring.

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]
NAB	2.70	192 → 162	192 → 106
NAB-d_4		196 → 166	-
NAT	2.65	190 → 160	190 → 79
NAT-d_4		194 → 164	-
NNK	2.5	208 → 122	208 → 148
NNK- d_7		215 → 126	215 → 152
NNK- d_4 (IS)		211 → 122	-
NNN	2.4	178 → 148	178 → 120
NNN- d_4		182 → 152	-

4.2.4.4 Validation

Assay validation was carried out according to FDA guidelines for bioanalytical methods validation [180]. The following validation criteria were investigated: selectivity, linearity, range, accuracy, precision, recovery, matrix effect and stability. The selectivity was proven by different experiments. First, 6 different filters were checked for interference from the analyte and the internal standard. There was no interference on the retention time of either the analyte or the internal standard. In addition, accuracy was determined at LLOQ, which ranged from 87 to 117 % for all analytes. Peak identity is confirmed by comparing the quantifier/qualifier ratio in six different matrix samples. For this purpose, the ratio described after determination from the calibration standards is compared with that of several selected matrix samples. The ratios determined differed less than 25 % from the comparison value from standard solutions. Additionally, matrix effect was not observed.

The calibration range is between 2.5 and 1000 pg per filter. To determine the limit of detection, 3 different filters were spiked at the LLOQ and the signal-to-noise (S/N) ratio was calculated using the analysis software (Analyst®, Sciex). The averaged concentration at a calculated S/N=3 is defined as the LOD. The theoretical limit of quantification (LLOQ) is calculated from a signal-to-noise ratio of 9 (3 x LOD). The LLOQs of this method are 2.5 pg per filter for all analytes. Carryover effect was not observed at 1000 pg per filter.

The precision was determined by spiking all analytes onto the filters at 4 different concentrations (LLOQ (S1), L (S2), M (S5), H (S8)). For precision on one day ("intraday"), five filters per level were freshly prepared and processed separately. This was repeated on three days ("interday"). The coefficients of variation of the intraday and interday precision determinations are below 10.3 % (at LLOQ: 14.7 %) (intraday) and below 10.0 % (at LLOQ: 10.6 %) (interday) for all analytes.The accuracy was determined for all analytes analogous to the precision. The criteria for accuracy (medium and high level: 85 - 115 %, low level at LLOQ: 80 - 120 %) were met for all analytes and in the respective concentration ranges and were between 98 % and 111 % for the medium and high levels and between 94 % and 109 % for the low levels.

The recovery was determined at 3 different concentration levels (5.0, 50.0 and 500.0 pg per filter). The recovery was consistent over the complete concentration range for each analyte, but it varies between the different analytes (NAB: 25 %; NAT: 28 %; NNN and NNK: 10 %).
Four different tests are used to determine the stability of the analytes: the short-term stability test, the freeze-thaw stability test, the stability of the stock solutions and the post-preparative stability. Two matrix samples of different concentrations (5.0 and 500 pg per filter) are used to determine the sample parameters. The accuracy of the determinations should be between 85 % and 115 % (80 % - 120 % to 3 x LLOQ). The short-term stability is determined at room temperature and a storage time of about 16 h, which is supposed to simulate a maximum residence time of the sample during work-up at room temperature and during collection. All analytes show sufficient stability for up to 16 h at room temperature. Freeze-thaw stability is determined using 6 filters (3 x 5.0 and 3 x 500.0 pg per filter) for one freeze-thaw cycle. Stabilities for all analytes were confirmed for one freeze-thaw cycle. Post-preparative stability verifies the stability of the analytes after work-up. For this purpose, the processed sample was left in the autosampler for 2 and 14 days and then reinjected. For all analytes, except NNK, stability is shown over 14 days in the autosampler. NNK is stable for only 2 days in the autosampler due presumably to D-H exchange of the internal standard. The stability of the stock solutions in methanol showed a stability of several years for both the analyte and the internal standard. The detailed validation protocol is summarized in appendix A in Table 21 and 22.

4.3 Biomarker analysis

Three new methods were developed during this project and three existing methods were adapted for the labeled metabolites. The newly developed methods are described in detail and the summarized validation data are presented at the end of each section (the detailed validation results are shown in Appendix A). For the existing methods, there are method summaries and the adaption presented including the additional mass transitions and the adaption of the sample preparation. All of the seven methods were fully validated methods according the FDA Guideline 2013 [179] or the FDA Guideline 2018 [180].
The measurement was also conducted according to the FDA Guidelines [179, 180]. This includes the measurement of a calibration with at least 6 non-zero calibrators levels (plus a blank (S00) and a zero (S0)) per analytical run, a sufficient amount of QC samples (5 % of the unknown study samples at 3 levels, but at least 6 in each analytical run) and washes. Further details regarding the acceptance criteria and the amount of samples are described in Section 2.5.

4.3.1 Nicotine and its metabolites

4.3.1.1 Urinary metabolites

The complete set of urine samples from Part A and B of the clinical study was used for analysis to show the excretion kinetic of nicotine (Part A) and for comparing controlled use and ad libitum use (Part B). In total 980 urine samples were analysed using the following summarized method.

4.3.1.1.1 Chemicals

Cotinine ($\geq$ 98 %), nicotine ($\geq$ 99 %), ammonium formate (for HPLC, $\geq$99 %) and ammonium hydroxide solution ($\geq$25 % in water) were purchased from Sigma (Taufkirchen, Germany). (3'R, 5'S)-3'-hydroxycotinine-O-β-d-glucuronide , (3'R, 5'S)-3'-hydroxycotinine-O-β-d-glucuronide (N-CD_3) were from Syntheselabor Dr. Mark (Worms, Germany). Cotinine-methyl-d_3 ($\geq$ 98 %), (3'R, 5'S)-3'-hydroxycotinine ($\geq$ 99 %), trans-3'-hydroxycotinine methyl-d_3 ($\geq$ 95 %), nicotine-N-β-glucuronide ($\geq$ 98 %), nicotine-N-β-glucuronide methyl-d_3 ($\geq$ 96 %), cotinine-N-β-glucuronide ($\geq$ 98 %), cotinine-N-β-glucuronide methyl-d_3 ($\geq$ 98 %), 4-hydroxy-(3-pyridyl)-butanoic acid ($\geq$ 98 %), 4-hydroxy-(3-pyridyl)-butanoic acid-d_4 ($\geq$ 98 %), (1'S,2'S)-nicotine 1'-oxide ($\geq$ 98 %), (1'S, 2'S)-nicotine 1'-oxide methyl-d_3 ($\geq$ 98 %), (S)-cotinine-1'-oxide ($\geq$ 98 %), (S)-cotinine-1'-oxide methyl-d_3 ($\geq$ 98 %), (R,S)-nornicotine ($\geq$ 97 %) and (R,S)-norcotinine ($\geq$ 98 %) were obtained from Toronto Research Chemicals (Ontario, Canada). Nicotine-d_3 ($\geq$ 96 %) was from CDN Isotopes Inc. (Quebec, Canada). Ammonium acetate (ULC/MS grade) and methanol (ULC/MS grade) were obtained from Biosolve BV (Valkenswaad, Netherlands). Methanol (for trace analysis) was purchased from Th. Geyer (Renningen, Germany). Oasis MCX cartridges (60 mg, 3 ml) were obtained from Waters Corp. (Milford, MA, USA).

4.3.1.1.2 Instrument and software

A Shimadzu Nexera X2 UPLC system including a binary pump, an autosampler, a degasser and a column oven (Shimadzu Corp., Kyoto, Japan) and a triple quadrupole mass spectrometer QTRAP®

6500$^+$ equipped with a Turbo V ion spray source, operating in positive ESI mode, was used for detection (AB Sciex, Darmstadt, Germany). High purity nitrogen (purity: 99.7 %) was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).
For the generation and the processing of the data Analyst® 1.6.3 (Sciex, Darmstadt, Germany) was used. The data evaluation was conducted with Office 2013 (Microsoft, Redmond, US) and Graph Pad Prism 8 (San Diego, US)

4.3.1.1.3 Method adaption

Nicotine and its 10 major metabolites in urine were determined according to a published and fully validated method of Piller et al. [74]. The quantification of nicotine-d_7 and its labeled metabolites required some minor modifications, including that 1 ml (instead of 0.5 ml) urine was used. Furthermore the internal standards for the nornicotine and norcotinine were replaced by nicotine-d_3 and cotinine-d_3, respectively, because nicotine-d_7 yields nornicotine-d_4 and norcotinine-d_3. The solid phase extraction using the Oasis MCX cartridges is fully described in [74]. The extract was evaporated to dryness and reconstituted in methanol. To improve the sensitivity the analysis was performed on a QTRAP® 6500$^+$ in positive electrospray ionization mode. The gradient elution and mass transitions of the unlabeled substances were kept according to Piller et al. [74].

Table 5: Mass transition and retention time (RT) of labeled nicotine and its 10 metabolites. MRM: multiple reaction monitoring, LLOQ: lower limit of quantification.

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	LLOQ [ng/ml]
Nicotine-d_7	7.3	170 → 135	170 → 133	1.5
Cotinine-d_7	6.4	184 → 84	184 → 101	0.51
OH-Cotinine-d_7	5.7	200 → 84	200 → 137	0.63
Nornicotine-d_4	5.6	153 → 134	153 → 136	1.20
Norcotinine-d_4	6.0	167 → 84	-	0.63
Nicotine-N-glucuronide-d_7	4.9	346 → 170	-	0.51
Cotinine-N-glucuronide-d_7	2.5	360 → 184	-	0.99
OH-Cotinine-N-glucuronide-d_7	4.9	376 → 200	-	1.50
Nicotine-N-oxide-d_7	5.3	186 → 133	186 → 121	0.21
Cotinine-N-oxide-d_7	4.8	200 → 84	200 → 102	1.50
Hydroxy-(pyridyl)-butanoic acid-d_4	4.3	182 → 152	-	0.63

Additionally, the mass transition of the metabolites for nicotine-d_7 were implemented into the existing method (Table 5). These mass transitions were defined by clarifying the fragmentation pattern of the unlabeled and replacing the hydrogen with deuterium. The area of the unlabeled and labeled analytes were normalized to the area of the corresponding signal of their d_3-labeled analogues. The

labeled analytes were evaluated with the calibration slope of the unlabeled analytes. This was validated by comparing the calibration slopes of nicotine and nicotine-d_7, nornicotine and nornicotine-d_4 as well as norcotinine and norcotinine-d_3. The calibration slopes of each analyte pair was within the acceptances criteria ($\pm$ 15 %).
The calibrations were performed using the the standard addition method with weighting. For this purpose, increasing amounts of the unlabeled standard compound were spiked into analyte-free matrix pool urine. The calibration ranges from 1.5 to 5000 ng/ml.

4.3.1.2 Nicotine and metabolites in plasma and saliva

The biological samples (plasma and saliva) from both Parts (A and B) were used for analysis of the pharmakokinetic of nicotine and cotinine in plasma. Additionally, the ratio between the plasma cotinine concentration and the saliva cotinine concentration were compared between the smokers and the vapers. For this purpose, the plasma and saliva samples were selected resulting in 1432 samples.

4.3.1.2.1 Chemicals

Cotinine ($\geq$ 98 %), nicotine ($\geq$ 99 %) sodium hydroxide ($\geq$ 99 %) and ammonium formate (for HPLC, $\geq$99 %) were purchased from Sigma (Taufkirchen, Germany). Cotinine-methyl-d_3 ($\geq$ 98 %), (3'R, 5'S)-3'-hydroxycotinine ($\geq$ 99 %), trans-3'-hydroxycotinine methyl-d_3 ($\geq$ 95 %) were obtained from Toronto Research Chemicals (Ontario, Canada). nicotine-d_3 ($\geq$ 96 %) was obtained from CDN Isotopes Inc. (Quebec, Canada). Formic acid (ULC/MS grade) and acetonitril (ULC/MS grade) were obtained from Biosolve BV (Valkenswaad, Netherlands). Ethyl acetate (for trace analysis) was purchased from Th. Geyer (Renningen, Germany).
For sample cleanup, different solutions were prepared. These were a 6 M sodium hydroxide solution and a 100 mM ammonium formate buffer (pH = 3.5) as eluent.
Additionally, four differently concentrated working solutions (AL 1: 1 ng/ml N; 5 ng/ml (OH)-Cot; AL 2: 10 ng/ml N; 50 ng/ml (OH)-Cot; AL 3: 100 ng/ml; N; 500 ng/ml (OH)-Cot; AL 4: 1 µg/ml N; 5 µg/ml (OH)-Cot) were prepared for the calibration. The IS-Mix containing 20 ng/ml N-d_3, 50 ng/ml (OH)-Cot-d_3 was prepared from methanol stock solutions.

4.3.1.2.2 Instrument and software

The utilized UPLC-MS/MS system consists of the Xevo TQ-S Triple Quadrupol mass spectrometer with electrospray ionization and the Acquity UPLC I-Class System with the modules: binary solvent manager, sample manager, sample organizer and column manager (Waters, Eschborn, Germany). High purity nitrogen (purity: 99.7 %) (was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany) and the argon collision gas was provided by a compressed gas cylinder (Linde Gas, Pullach, Germany).

4.3.1.2.3 Sample preparation and analytical methodology

Labeled and unlabeled nicotine, cotinine in plasma as well as labeled and unlabeled cotinine and

3'-trans-hydroxcotinine (OH-Cot) in saliva were determined by means of an UPLC-MS/MS based on [79]. To 100 µl plasma or saliva, 20 µl IS-Mix and 4 µl 6 M sodium hydroxide were added. The mixture was extracted with 1 ml ethyl acetate for 30 min. Subsequently the samples were centrifuged (15000 rpm, 4 °C, 15 min) and 900 µl of the supernatant were evaporated almost to dryness. The residue is reconstituted in 100 µl acetonitrile, of which 10 µl were injected into the UPLC-MS/MS system. Chromatographic separation was performed on an Acquity UPLC BEH HILIC column (2.1 x 150 mm, 1.7 µm; Waters, Eschborn, Germany). The elution was isocratic with 100 mM ammonium formiate buffer (pH = 3.5, eluent A) and acetonitril (eluent B) with a ratio of 20 % A and 80 % B. The column was isotherm at 40 °C with a flow of 0.4 ml/min. The source parameters were optimized to capillary voltage 3000 V, source offset 30 V, desolvation temperature 350 °C, desolvation gas 650 l/h, cone gas 150 l/h and the nebulizer 7 bar. Analytes and IS were ionized in the ESI positive mode. For quantification, the following mass transitions were utilized: m/z 163 → 132 (nicotine), m/z 177 → 80 (cotinine), m/z 193 → 80 (OH-Cot), m/z 170 → 135 (nicotine-d_7), m/z 284 → 84 (cotinine-d_7), m/z 200 → 84 (OH-Cot-d_7), m/z 166 → 130 (nicotine-d_3), m/z 180 → 80 (cotinine-d_3), m/z 196 → 80 (OH-Cot-d_3). For the labeled and unlabeled analytes LLOQs were 0.1 ng/ml for nicotine and 0.5 ng/ml for cotinine and OH-cotinine. The calibrations were performed using the the standard addition method with weighting. For this purpose, increasing amounts of the unlabeled working solutions were spiked in ultrapure water. Water was used as a replacement because there was no analyte-free matrix available. The calibration ranges from 0.1 to 100 ng/ml for nicotine and from 0.5 to 500 ng/ml for cotinine and hydroxy-cotinine.

4.3.1.2.4 Validation

Assay validation was carried out according to FDA guidelines for bioanalytical methods validation [180]. The following validation criteria were investigated: selectivity, linearity, range, accuracy, precision, recovery, matrix effect and stability.

The specificity of the method was ensured by the fact that no interfering signals at the expected RT of the standard and internal standard were found and that the spiked amounts at LLOQ were found in the matrix within an accuracy of 80 and 120 %.

Intra- and interday precision along with the accuracies were tested at 4 different concentration levels at 3 different days. The precisions across all analytes were better than 8 % (at LLOQ: 16 %). The accuracies of the high and medium levels were within the range 95 - 114 % and for the low levels between 86 and 113 %.

The LODs and LLOQs were defined as S/N ratios 3:1 and 9:1, respectively. The LODs were calculated by the software using the signal-to-noise algorithm (N: 0.02 ng/ml, Cot: 0.003 ng/ml and OH-Cot: 0.02 ng/ml) The LLOQs are more than a factor 3 high than the LODs due to the background contamination from the environment and the used material. The LLOQ was set to 0.1 ng/ml for N and 0.5 ng/ml for Cot and OH-Cot, respectively.

The recovery rate was for N and Cot between 59 % and 67 % and for OH-Cot between 29 and 39 %. The recovery was determined by comparing the extract from the plasma/saliva samples and the stock solutions in ACN with the expected concentration. In both matrices (aerosol/smoke) was not a matrix effect detected.

The calibration ranged from 0.1 ng/ml to 100 ng/ml for N and from 0.5 to 500 ng/ml for Cot and OH-Cot which. No carryover was observed from the highest calibrator to a blank sample.
The stability was characterized by means of the short-term, long-term stability of the analytes in matrix and post-preparative stability as well as six freeze-thaw cycles. All analytes were found to be stable for 16 h at room temperature. The post-preparative stability was even proven for 5 days in the autosampler (10 °C). The freeze-thaw cycles were tested with the unprepared samples for six cycles and it was found that all analytes are stable. The long-term stability for N and Cot in matrix were proven for 14 months. The stability of the stock solution was given for 17 months for N and Cot.
The detailed validation protocol is summarized in Appendix A Table 24.

4.3.2 Propylene glycol and Glycerol

The samples (urine, plasma and saliva) from Part A an B of the clinical study were used for analysis of the time-dependent uptake and excretion in different matrices. For this reason, 2410 samples were analyzed using the method described below.

4.3.2.1 Chemicals and solutions

Propylene glycol ($\geq$ 99.5 %), glycerol ($\geq$ 99 %), glycine ($\geq$ 99 %), sodium hydroxide ($\geq$ 98 %) and benzoyl chloride (Reagent Plus, $\geq$ 99 %) were purchased from Sigma (Taufkirchen, Germany). Glycerol-d_5 ($\geq$ 99 %) and propylene glycol-d_6 ($\geq$ 99 %) were obtained from CDN Isotopes Inc. (Quebec, Canada). $^{13}C_3$-Glycerol (99 %) and $^{13}C_3$-propylene glycol ($\geq$ 96 %) were obtained from Synthèse Synthèse AptoChem (Montréal, Canada). Formic acid (ULC/MS grade) and acetonitril (ULC/MS grade) were purchased from Biosolve BV (Valkenswaad, Netherlands). Pentan (Chromanorm®) was obtained from VWR.
For sample cleanup, different solutions were prepared. These were a 4 M sodium hydroxide and a 10 % glycine solution.
Furthermore, the stock solutions (SL) were prepared by weighing in each standard and internal standard and dissolve them in ethanol. Further dilution for the working solutions AL 1 (250 µg/ml) and AL 2 (10 µg/ml) were made in ultrapure water. PG-d_6 and G-d_5 were also further diluted with ultrapure water to a concentration of 5 µg/ml for the IS-Mix.

4.3.2.2 Instrument and software

The LC-MS/MS system consists of the HPLC system HP 1100 (Agilent Technologies, Waldbronn, Germany) with the following modules: binary high pressure pump, column oven and degasser; the autosampler HTC PAL (Axel Semrau, Sprockhövel, Germany) and tandem quadrupol mass spectrometer API 4000™(Sciex, Darmstadt, Germany). High purity nitrogen (purity: 99.7 %) was produced by a nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany). For the generation and the processing of the data Analyst® 1.5.2 (Sciex, Darmstadt, Germany) was used.

4.3.2.3 Sample preparation and analytical methodology

The determination of PG and G in plasma, urine and saliva is based on the Schotten-Baumann re-

action [189, 190]. The Schotten-Baumann method in aqueous solution using a strongly alkaline medium is well described for the benzoylation of glycols [191–194]. In this study the derivatization procedure was also used for trivalent alcohol glycerol. The benzoyl derivatives were prepared and extracted following the methods in the previous papers [194] with some modifications. 10 µl IS-Mix was spiked to 25 µl of biological sample. 500 µl 4 M sodium hydroxide solution, 100 µl benzoyl chloride and 2 ml pentane were added to the samples and mixed thoroughly for 15 min using a multishaker. To neutralize the excess of the derivatization agent 500 µl glycine solution (10 % w/w) was added and agitated again for 15 min. Before transferring the supernatant (pentane layer), the sample was centrifuged (10 min. 4000 rpm, 10 °C). The pentane extract was evaporated to dryness. The residue was taken up in 100 µl ACN. 10 µl of the sample was injected in the LC-MS/MS system. The separation was performed on a on a Kinetex® 5 µm EVO C18 column (150 x 2.1 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) setting the flow rate to 1.0 ml/min and the oven temperature to 40 °C. The gradient elution was conducted with a mobile phase water with 0.1 % formic acid (eluent A) and ACN (eluent B) (0-0.6 min: 50 % B; 0.7-2 min: 40 % B; 2.1-4.0 min: 20 % B; 4.1-6.0 min: 5 % B; 6.1-8.0 min: 50 % B). The tandem mass analysis was performed on the triple quadrupole MS (API 4000, Sciex, Darmstadt, Germany) in positive mode with an electrospray ionization (ESI) using the following parameter of the Turbo V Ion Source: curtain gas: 2.1 bar, source gas 1; 4.1 bar, source gas 2: 3.4 bar, ion spray voltage: 5500 V, source temperature: 300 °C and collision gas: 0.7 bar. Labeled and unlabeled analytes as well as their corresponding internal standards were monitored in scheduled MRM mode (sMRM, Table 6) with a detection window of 60 sec.
The calibrations were performed using the the standard addition method with weighting. For this purpose, increasing amounts of the unlabeled standard compound were spiked in water. The calibration ranges from 0.1 to 150 µg/ml. LLOQs for the ^{13}C-labeled PG and G were 0.10 and 0.05 µg/ml, respectively. LLOQs for the unlabeled PG and G were 0.1 µg/ml.

Table 6: MS/MS-parameters for the labeled and unlabeled propylene glycol (PG) and glycerol (G) and their corresponding internal standards (IS). RT: retention time, MRM: multiple reaction monitoring.

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	IS	IS MRM [m/z → m/z]
PG	2.1	285 → 163	285 → 105	d_6-PG	291 → 169
$^{13}C_3$-PG		288 → 166	288 → 105		
G	3.1	405 → 283	405 → 105	d_5-G	410→ 288
$^{13}C_3$-G		408 → 286	408 → 105		

4.3.2.4 Validation

Assay validation was carried out according to FDA guidelines for bioanalytical methods validation [179] with minor modifications, mainly due to the lack of analyte-free matrix. The following validation criteria were investigated: selectivity, linearity, range, accuracy, precision and stability.
The method shows high selectivity by using the LC-MS/MS technique, since at least one specific

mass transition is measured for each analyte. Peak identity is confirmed by comparing the quantifier/qualifier ratio in six different matrix samples. For this purpose, the described ratio in the matrix is compared to the ratio of the calibration standards in water. The determined ratios did not deviate significantly from those of the standard solutions (< 25 %). At the same time, the mass transitions of the analogous internal standards were checked for interference in matrix samples. No interference was observed. To confirm selectivity, matrix samples of different origins (N=6) are spiked at a medium concentration. The accuracy of the spiking was between 89.6 and 108.0 %.
Since no analyte-free matrix is available, the limit of detection (LOD) is determined by measuring 3 different matrix samples whose signal-to-noise ratio should not exceed the value 30. The signal-to-noise ratio is calculated using the script "S/N using 3 x Std Deviation" of the Analyst software version 1.5.2 (Sciex,Darmstadt, Germany). The theoretical limit of quantification (LLOQ) is calculated from a signal-to-noise ratio of 9 (3 x LOD). The verification and thus the determination of the actual LLOQ is done by measuring three different matrix samples concentrated at the LLOQ, whose precision (CV) in a 6-fold determination must be within ± 20 %. In addition, a concentration corresponding to the LLOQ is then spiked onto these samples. The accuracy of this addition must be between 80 and 120 % in a 6-fold determination. The concentration of the smallest calibrator should also be selected so that it corresponds to the LLOQ. For this method the following confirmed LLOQs were obtained: PG: 0.1 µg/ml; G: 0.1 µg/ml. The linearity of the method was determined by calibration experiments in water. The calibration curve ranged from 0.1 - 100 µg/ml. A carryover effect was not observed until a concentration of 150 µg/ml.
Serial ("intraday") and day-to-day ("interday") precision were performed for all analytes in human urine and plasma, respectively. For serial precision, urine or plasma pools were used and analyzed five times each at three different concentrations. Day-to-day precision was measured by single determination (diluted, native or spiked with standard solutions) on six different days (criteria: medium and high level: CV $\leq$ 15 %, low level up to a maximum of 3 x LLOQ: CV $\leq$ 20 %). The coefficients of variation of the intraday precision determination ranged from 2.0 to 18.0 % for both analytes. The coefficients of variation of the interday precision determination range from 3.1 to 15.2 % for both analytes. The accuracy was determined for all analytes in water, for this purpose the water was spiked with standard solutions at three different concentrations and analyzed five times each. The criteria for accuracy (medium and high level: 85 - 115 %, low level at LLOQ: 80 - 120 %) were met for both analytes and in the respective concentration ranges and were between 92.0 and 106.4 %. For the 5-fold determination of accuracy, the same CV tolerances apply as for precision. These were also met.
Four different tests were conducted to determine the stability of the analytes: short-term, long-term, freeze-thaw, and post-preparative stability. In addition, the stability of the stock solutions is documented. Two matrix samples of different concentrations (one low level, one high level) are used to determine the sample parameters. The determined accuracy must be between 85 and 115 %, and between 80 and 120 % for concentrations $\leq$ 3 x LLOQ. Short-term stability is determined at room temperature and a storage period of at least 16 h. This is intended to simulate a maximum storage time of the sample during processing at room temperature and during urine collection. All analytes showed stability at room temperature for up to 24 h. Freeze-thaw stability is determined with the

same sample processed and measured in triplicates after thawing twice, four times and six times. PG and G show stability over up to 6 freeze-thaw cycles. Post-preparative stability provides an indication of the stability of the final extracts under real storage conditions in the autosampler (approximately 10 °C). For this purpose, the extracts are immediately measured after sample preparation and then they remain in the autosampler. Both analytes did not show any changes in concentration after storage for 9 days in the autosampler (10 °C). The stock solutions in water are stable for 24 months. The long stabilities of matrix samples vary between 8 months and 21 months depending on the analyte and the matrix.
The detailed validation protocol is summarized in Appendix A Table 23.

4.3.3 Mercapturic acids

The mercapturic acids represent the uptake of the carbonyls, acrolein and crotonaldehyde, and the epoxides, ethylene oxide, propylene oxide and glycidol, which are associated among other things with smoking. To compare the amount of smoke-associated and vape-associated carbonyls/epoxides with each other, 669 urine samples were analysed. The 669 samples include the samples of the first 48 hours of Part A and the whole set of Part B.

4.3.3.1 Chemicals

N-Acetyl-S-(3-hydroxypropyl)cysteine (3-HPMA), $^{13}C_3$-^{15}N-N-acetyl-S-(3-hydroxypropyl)cysteine (3- $^{13}C_3$- ^{15}N-HPMA), and N-acetyl-S-(2-hydroxypropyl)cysteine (2-HPMA) were purchased from Synthèse AptoChem (Montreal, Canada). N-Acetyl-S-(2-hydroxypropyl)cysteine-d_3 dicyclohexylammonium salt (2-HPMA-d_3), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine-d_7 (DHBMA-d_7), N-acetyl-S-(2-hydroxyethyl)-L-cysteine sodium salt (HEMA), N-acetyl-S-(2-hydroxyethyl-d_4)-L-cysteine (HEMA-d_4), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine dicyclohexylammonium salt (HMPMA), N-acetyl-d_3-S-(3-hydroxypropyl-1-methyl)-L-cysteine dicyclohexylammonium salt (HMPMA-d_3) were obtained from Toronto Research Chemicals (Ontario, Canada). Acetic acid, ammonium formate, formic acid, and sodium hydroxide were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetonitrile with 0.1 % formic acid (ULC/MS grade), acetonitrile (ULC/MS grade) and ammonium acetate (ULC/MS grade) were purchased from Biosolve BV (Valkenswaad, Netherlands).

4.3.3.2 Instrument and software

Method 1: The LC-MS/MS system consists of the two HPLC systems and tandem quadrupol mass spectrometer API 5000™ (Sciex, Darmstadt, Germany) equipped with a Turbo V™. The first HPLC system, a HP 1200 (Agilent Technologies, Waldbronn, Germany) with the modules: binary high pressure pump, column oven and degasser; the HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) was used for the gradient elution and the second system, a HP 1100 (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a degasser for the online purification step.
Method 2: Liquid chromatography was performed with a Nexera X2 System including a binary pump, an autosampler and a column oven (Shimadzu Corp., Kyoto, Japan). A triple quadrupole mass

spectrometer QTRAP® 6500+ (Sciex, Darmstadt, Germany) equipped with a Ion Drive™Turbo V source, operating in negative ESI mode, was used for detection. Both methods used high purity nitrogen (purity: 99.7 %) which was produced by the nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany). For the generation and the processing of the data Analyst® 1.5.2 and 1.6.3 (Sciex, Darmstadt, Germany) were used.

4.3.3.3 Method adaption

The determination of the mercapturic acids is published by Pluym et al. [195] and divided into two different methods.

Method 1 (MA I, comprising among other MAs HEMA and HMPMA) includes an online purification step by means of column-switching. Urine samples are injected into the LC-MS/MS-system after dilution. Online-extraction using a restricted access material phase (LiChrosphere reversed-phase C8, 25 µm, Merck, Darmstadt, Germany) for clean-up and enrichment is followed by chromatographic separation on a Luna C8 (2) column (150 x 4.6 mm, 3 µm, Phenomenex, Aschaffenburg, Germany). The method was fully validated for 10 MAs and published in 2015 [195]. The LLOQ for the labeled and unlabeled analytes are: ($^{13}C_2$-)HEMA): 0.2 ng/ml; ($^{13}C_4$)-HMPMA: 5.0 ng/ml.

Table 7: Mass transitions of the labeled and unlabeled mercapturic acids and their internal standards (IS). MRM: multiple reaction monitoring, ($^{13}C_3$-)2,3-dihydroxypropylmercapturic acid (($^{13}C_3$-)DHPMA), ($^{13}C_3$-)3-hydroxypropylmercapturic acid (($^{13}C_3$-3-HPMA)), ($^{13}C_3$-)2-hydroxypropylmercapturic acid (($^{13}C_3$-)2-HPMA), ($^{13}C_{2/4}$-)hydroxymethylpropylmercapturic acid (($^{13}C_{2/4}$-)HMPMA), ($^{13}C_2$-)2-hydroxyethylmercapturic acid (($^{13}C_3$-)HEMA).

Method	Analyte	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	IS	IS MRM [m/z → m/z]
MA I	HEMA	206 → 77	206 → 75	HEMA-d_4	210 → 81
	$^{13}C_2$-HEMA	208 → 79	208 → 77		
	HMPMA	234 → 105	234 → 103	HMPMA-d_3	237→ 105
	$^{13}C_2$-HMPMA	236 → 107	236 → 105		
	$^{13}C_4$-HMPMA	238 → 109	238 → 107		
MA II	2-HPMA	220 → 91	220 → 89	2-HPMA-d_3	223 → 91
	$^{13}C_3$-2-HPMA	223 → 94	-		
	3-HPMA	220 → 91	220 → 89	$^{13}C_3$-^{15}N-3-HPMA	224→ 91
	$^{13}C_3$-3-HPMA	223 → 94	-		
	DHPMA	236 → 107	236→ 128	DHBMA-d_7	257→ 78
	$^{13}C_3$-DHPMA	238 → 107	238→ 130		

The mercapturic acids 2-HPMA, 3-HPMA, and DHPMA are measured with Method II (MA II) by LC-MS/MS after evaporation of the urine sample and resuspension in 100 µl methanol. 2 µl of the resuspension was injected onto an Acquity UPLC BEH C18 column (150 x 3 mm; 1.7 µm, Waters,

Eschborn, Germany). The method was fully validated for 8 MAs and published in 2015 [195]. DHPMA was implemented into the existing method MA II, since glycidol (the precursor for DHPMA) was reported as a pyrolysis product of glycerol [25] (see Table 1). DHBMA was used as internal standard for DHPMA because the commercially available internal standard was $^{13}C_3$-DHPMA which is also the labeled analyte of interest. The method MA II was transferred on an UHPLC-MS/MS system (QTRAP 6500+/Shimadzu Nexera) in order to improve sensitivity for the intended purpose resulting in the following LLOQs: ($^{13}C_3$-)2-HPMA: 0.5 ng/ml; (3-HPMA): 10 ng/ml; ($^{13}C_3$-3-HPMA): 0.5 ng/ml; (DHPMA): 10 ng/ml; ($^{13}C_3$-DHPMA): 0.8 ng/ml.
The mass transitions for both methods of the labeled analytes were selected according to the published fragmentation pathways of mercapturic acids [196] and they are summarized in Table 7.

4.3.4 Biomarker of formaldehyde and acetaldehyde

4.3.4.1 Chemicals and solutions

2-Methyl-thiazolidine-4-carboxylic acid (98 %) was obtained from Santa Cruz Biotechnology (Dallas,TX, USA). L-thiazolidine-4-carboxylic acid (98 %), $^{13}C_3$-^{15}N-L-cysteine (98 %), Cys-Gly ($\geq$ 85 %), hydrochloric acid ($\geq$ 37 %), propyl chloroformate (98 %), isooctane ($\geq$ 99.5 %), n-propanol ($\geq$ 99.9 %), anhydrous pyridine (99.8 %), formaldehyde solution (37 wt. % in water), acetaldehyde ($\geq$ 99.5 %) and ammonium formate for trace analysis were purchased from Sigma (Taufkirchen, Germany). Chloroform for residue analysis was from Promochem (Wesel, Germany). Methanol for residue analysis was obtained from Th. Geyer (Renningen, Germany). Formic acid (ULC/MS grade), methanol (ULC/MS grade) and acetonitrile (ULC/MS grade) were purchased from Biosolve BV (Valkenswaad, Netherlands). Ultrapure water was prepared by arium® pro ultrapure water system (Sartorius, Göttingen, Germany). Oasis MCX cartridges (150 mg, 6 ml) were obtained from Waters Corp. (Milford, MA, USA).
The internal standards $^{13}C_3$-^{15}N-thiazolidine-4-carboxylic acid (TCA) and $^{13}C_3$-^{15}N-2-methyl-thiazolidine-4-carboxylic acid (MTCA) as well as the dipeptides thiazolidine-4-carbonyl-glycine (TCG) and 2-methyl-thiazolidine-4-carbonyl-glycine (MTCG) were self-synthesized. The synthesis was performed according to Anni et al. [156]. Briefly, the $^{13}C_3$-^{15}N-cysteine or the cysteinylglycine was dissolved in 10 mM phosphate puffer (pH = 7.4). To the reaction mixture, FA and AA, respectively, was added and incubated at 37 °C for one hour. The samples were deep frozen (- 80 °C) and subsequently lyophilized. The received conjugate was purified by means of two different high-performance liquid chromatography methods coupled to UV/Vis detector. The structural analysis of the products were performed by electrospray ionization and an ion trap mass spectrometer (QTRAP® 6500$^+$, Sciex). The purity was determined by UV/VIS.
For sample cleanup, different solutions were prepared. The propyl chloroformate solution contained 17.4 % propyl chloroformate, 11 % isooctane and 71.6 % chloroform.The concentration of the standards were corrected by the purity of the standards. The working solutions were prepared in water at 4 different concentrations: 100 µg/ml, 5 µg/ml, 200 ng/ml and 10 ng/ml for TCA and MTCA as well as 200 µg/ml, 10 µg/ml, 400 ng/ml and 20 ng/ml for TCG and MTCG. The IS-Mix had a concentration of 100 ng/ml of $^{13}C_3$-^{15}N-TCA and $^{13}C_3$-^{15}N-MTCA in water.

4.3.4.2 Instrument and software

Liquid chromatography was performed with a Nexera X2 System including a binary pump, an autosampler and a column oven (Shimadzu Corp., Kyoto, Japan). A triple quadrupole mass spectrometer QTRAP® 6500+ (Sciex, Darmstadt, Germany) equipped with a Turbo V source, operating in positive ESI mode, was used for detection. High purity nitrogen (purity: 99.7 %) was produced by the nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).

4.3.4.3 Sample preparation and analytical methodology

Aliquots of 200 µl of plasma were mixed with 20 µl of IS-Mix and 500 µl water. The derivatization was performed by adding 300 µl n-propanol/pyridine (4:1; v/v) and 200 µl propyl chloroformat-solution to the sample mixture, followed by mixing thoroughly. 2 ml isooctane are added and the samples are agitated for 5 min. After centrifugation (10 min, 4000 rpm, 10 °C) the upper organic phase was transferred into another vial and evaporated to dryness in a concentrator. The samples were reconstituted in 100 µl 100 mM ammonium acetate / methanol (95:5; v/v) and 10 µl of the solution was injected into the UPLC-MS/MS system.

Urine samples were prepared according to the plasma sample with some modifications. Prior to the derivatization step the urine samples were purified by solid phase extraction (SPE). Aliquots of 500 µl of urine were mixed with 50 µl of IS-Mix and the pH was reduced to approximately 1 by adding 4 µl fuming hydrochloric acid (HCl). SPE columns were preconditioned with 5 ml each methanol and 0.1 M HCl. The acidified urine samples were loaded on the SPE columns and then washed with 5 ml each 0.1 M HCl and methanol. Analytes were eluted with 2 ml methanol/ammonium hydroxide (90/10; v/v) under strong basic conditions (pH 12). The eluate was evaporated to dryness in a concentrator and reconstituted in 500 µl water. The derivatization was performed as described for the plasma samples above.

Table 8: Quantifier and qualifier mass transitions of (((methyl-)thiazolidine carboxylic acid ((M)TCA) and (methyl-)thiazolidine carbonyl glycine ((M)TCG) and the internal standards (IS) with their retention times (RT). MRM: multiple reaction monitoring.

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	IS	IS MRM [m/z → m/z]
TCG	2.5	319 → 174	319 → 88	$^{13}C_3$-^{15}N-TCA	266 → 177
^{13}C-TCG		320 → 175	320 → 89		
TCA	4.9	262 → 174	262 → 88		
^{13}C-TCA		263 → 175	263 → 88		
MTCG	3.7	333 → 188	333 → 102	$^{13}C_3$-^{15}N-MTCA	280→ 191
$^{13}C_2$-MTCG		335→ 190	335 → 104		
MTCA	6.2	276 → 188	276→ 102		
$^{13}C_2$-MTCA		278 → 190	278→ 102		

Chromatographic separation was achieved by gradient elution on a Kinetex ® 1.7 µm Phenyl-Hexyl (100 mm x 2.1 mm, Phenomenex, Aschaffenburg, Germany) with 0.1 % formic acid in water (eluent A) and a mixture of MeOH and ACN (3:7; v/v) with 0.1 % formic acid (eluent B) as mobile phases. Gradient elution started with 30 % B for 1 min, followed by a linear increase to 45 % until 6.5 min, a step to 100 % B within 0.01 min. 100 % B was held for 2.5 min before the column was re-equilibrated for 3 min at 30 % B. The flow rate was set to 0.75 ml/min throughout the entire run. The column oven was maintained at 50 °C. The turbo-ion spray source was operated in positive ionization mode using the following settings: ion spray voltage: 5500 V, ion source heater temperature: 600 °C, source gas 1: 4.8 bar, source gas 2: 5.5 bar, and curtain gas: 3.4 bar. The mass transitions were monitored in scheduled multiple reaction monitoring (sMRM) mode, applying a retention time window of 60 s. Quadrupoles Q1 and Q3 were operated in unit resolution. Quantifier and qualifier mass transitions and retention time are summarized in Table 8.
The calibrations were performed using the the standard addition method with weighing. For this purpose, increasing amounts of the working solutions were spiked in water. The calibration ranges from 0.5 to 200 ng/mL for (M)TCA and 1.0 to 400 ng/mL for (M)TCG, respectively.

4.3.4.4 Validation

Method validation was performed according to the FDA guidelines [180] for bioanalytical method validation with minor modifications, mainly due to the lack of analyte-free matrix. Method performance was evaluated by determining the following parameters.
Quantification was performed with the standard addition method. The calibration was obtained by spiking increasing amounts of analyte into water. Calibration slopes of water and urine were found to be in the similar range (water against urine CV: 5.9 % (TCA); 10.1 % (MTCA); 7.3 % (TCG); 14.6 % (MTCG) water against plasma CV: 3.5 % (TCA); 7.8 % (MTCA); 5.4 % (TCG); 11.3 % (MTCG)), proving the applicability of water as surrogate matrix.
The linear range was found to be from 0.5 to 200 ng/ml for (M)TCA and 1.0 to 400 ng/ml for (M)TCG, respectively. Least squares weighted (1/x) linear regression of the analyte/IS peak ratios was employed to determine the calibration curve (y = A + B * x), with the parameters: A as intercept, B as slope, y as peak area relative to IS and × the concentration (ng/ml) of the analyte. The mean coefficient of determination (r^2) was 0.998 $\pm$ 0.002 (N = 5) for the 4 different analytes. The LODs were defined as a signal-to-noise ratio of 3:1 and the resulting LLOQ were set to 0.5 and 1.0 ng/ml, respectively. LLOQs were verified in five replicates analysed on three different days with respect to the accuracy and precision.
The method specificity was determined by assessing the accuracy of spiked matrix samples as well as the Quan/Qual ratio for each analyte. The accuracy of the spiking was found to be in the range of 87 - 113 % across all analytes. The deviation of the Quan/Qual ratio between the aqueous solution and the matrix samples was below 25 % for all analytes in both matrices (plasma, urine). Intra- and interday accuracy in spiked matrix samples were found to be within 85 - 115 % and the precision expressed as the coefficient of variation (CV) was < 12 %.
The recovery rate was determined in urine which covers only the SPE-step due to the lack of purified

derivatized standard. The recovery rate ranged from 66 % for TCA and MTCA (CV < 15 %) and 33 % for MTCG and TCG (CV < 15 %). The matrix effect was determined by a post-column infusion experiment due to the lack of analyte-free matrix. Ion suppression by matrix interference was in the range of -33 % and -60 % for plasma and urine, respectively. However, it was demonstrate that the internal standards fully compensate for the ion suppression (data not shown). No carryover was observed from a highly concentrated standard to a blank sample.
The analytes proved to be stable under all testing conditions except for the long-term stability of MTCA. Stability experiments included 6 freeze-thaw cycles, post-preparative stability over 5 days in an autosampler (10 °C), short-term stability overnight at room temperature as well as the long-term stability. The long-term stability was conducted after 2 months and 21 months. TCA is stable until 21 months. In contrast, stability for MTCA could only be proven for 2 months, showing a decline to 60 % compared to the reference standard after 21 months.

4.3.5 Tobacco specific nitrosamines

The analysis of the TSNAs in urine samples was conducted with a subset of 75 samples. The subset included three samples of each subject: the morning urine, the urine spot after 24 h and the last urine spot of Part B (vaper) or the last urine spot of Part A (smoker).

4.3.5.1 Chemicals and solutions

N-Nitrosoanabasin (NAB, 98 %), N-Nitrosoanatabin (NAT, 98 %), N-Nitrosonornicotin (NNN, 98 %), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, 98 %) and 4-(Methyl-d3-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL-d_3, 98 %) were purchased from Toronto Research Chemicals (Ontario, Canada). Ammonium hydroxide solution (≥25 % in water), disodium hydrogen phosphate (≥99.5 %), potassium phosphate dibasic (≥98 %), heptane (Chromasolv® Plus for HPLC 99 %), hydrochloric acid (≥ 37 %), and toluene (Chromasolv® Plus for HPLC ≥99.9 %) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Dichloromethane (Picograde®) and hexane (Pico-grade®) were supplied by LGC GmbH (Wesel, Germany). Methanol (for residue analysis) was purchased from Th. Geyer (Renningen, Germany). β-Glucuronidase (E.Coli, 500 kUnits) were from Megazyme (Bray, Ireland). Acetonitrile with 0.1 % formic acid (ULC/MS grade) and ammonuim acetate (ULC/MS grade) were purchased from Biosolve BV (Valkenswaad, Netherlands). SupelMIP™TSNA SPE cartridge (50 mg, 10 ml) was obtained from Supelco (Bellefonte, USA). Oasis MCX cartridges (60 mg, 3 ml) were obtained from Waters Corp. (Milford, MA, USA).
For sample cleanup, different solutions were prepared. These were a 10 mM ammonium acetate buffer, a 0.1 M hydrochloric acid solution and a 0.67 M phosphate buffer (pH 7.2). Additionally the following working solutions and IS Mix were prepared: AL I: 10 µg/ml NAB, NAT, NNN, NNAL in water; AL II: 100 ng/ml NAB, NAT, NNN, NNAL in water; 10 ng/ml NAB, NAT, NNN, NNAL in water; 200 pg/ml; IS-Mix: 25 ng/ml NNAL-d_3 in ultrapure water.

4.3.5.2 Instrument and software

Liquid chromatography was performed with a Nexera X2 System including a binary pump, an au-

tosampler and a column oven (Shimadzu Corp., Kyoto, Japan). A triple quadrupole mass spectrometer QTRAP® 6500$^+$ (Sciex, Darmstadt, Germany) equipped with an Ion Drive™Turbo V source, operating in positive ESI mode, was used for detection. High purity nitrogen (purity: 99.7 %) was produced by the nitrogen generator NGM 22-LC/MS (cmc Instruments, Eschborn, Germany).
For the generation and the processing of the data Analyst® 1.6.3 (Sciex, Darmstadt, Germany) was used.

4.3.5.3 Method adaption

The method is a fully validated and well established method in our laboratory. Therefore the method will be only summarized briefly and the method adaption will be shown.
1.5 ml phosphate buffer (pH 7.2), 20 µl IS-Mix and 40 µl β-glucuronidase (155 units/µl) were added to 8 ml urine. After the incubation (37°C, over night) the samples were centrifuged for 20 min at 3300 g. The first of the two solid phase extractions were performed with the SupelMIP™TSNA SPE cartridges. The SPE cartridges were preconditioned with 10 ml 10 % MeOH in DCM, 1 ml MeOH and 1 ml water. The enzymatically cleaved urine sample was loaded onto the cartridge and was washed with 10 mM ammonium acetate buffer, 2 ml heptane and 1 ml hexane. After drying of the cartridge the sample was eluted with DCM/toluene (1:1, v/v). The eluate was dried in a concentration and the residue reconstituted in phosphate buffer (pH 7.2). The second solid phase extraction was performed with the Oasis MCX cartridges. The cartridges were preconditioned 2 ml MeOH, 1 ml 10 % ammonium hydroxide solution in MeOH and 2 ml water. The reconstituted samples were loaded to the SPE cartridges. The cartridges were washed with 2 ml water, 2 ml 0.1 N hydrochloric acid and 2 ml MeOH. The analytes were eluted with 2 ml 10 % ammonium hydroxide solution in MeOH and evaporated to dryness in a concentrator.

Table 9: Mass transition and retention time (RT) of labeled and unlabeled N-Nitrosoanabasin (NAB), N-Nitrosoanatabin (NAT), N-Nitrosonornicotin (NNN), 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), MRM: multiple reaction monitoring.

Analyte	RT [min]	Quantifier MRM [m/z → m/z]	Qualifier MRM [m/z → m/z]	LLOQ [pg/ml]	IS	IS MRM [m/z → m/z]
NAB	2.65	192 → 162	192 → 106	5.0	NNAL-d_3	213 → 183
NAB-d_4		196 → 166	-			
NAT	2.60	190 → 160	190 → 79	2.0		
NAT-d_4		194 → 164	-			
NNAL	2.12	210 → 180	210 → 93	2.0		
NNAL-d_7		217→ 187	-			
NNN	2.30	178 → 148	178→ 120	0.5		
NNN-d_4		182 → 152	-			

The residue was taken up in 100 µl MeOH/water (1:4, v/v) and 10 µl of the extract were injected on an Acquity UPLC HSS T3 column (100 mm x 2.1 mm, 1.8 µm, Waters, Eschborn, Germany).

The gradient elution was performed with water with 0.1 % ammonium acetate (pH 6.2 - 6.4, eluent A) and acetonitrile with 0.1 % formic acid (eluent B) following this gradient: 0-1 min: 10 % B , 1-2 min: 10 - 50 % B, 2-4 min: 50 % B and 4-7 min: 10 % B. The column was kept isotherm at 45 °C and with a flow of 0.6 ml/min.
The tandem mass analysis was performed on the triple quadrupol mass spectrometer QTRAP® 6500^+ in positive mode with an IonDrive™Turbo V source using positive electrospray ionization. The parameters of the source are summarized in the following: curtain gas: 2.4 bar, source gas 1: 3.1 bar, source gas 2: 3.1 bar, ionspray voltage: 5500 V, source temperature: 500 °C and collision gas: high. The labeled and unlabeled analytes were monitored in MRM mode. The internal standard for all analytes was NNAL-d_3 due to the fact that the other corresponding internals standard are potential degradation products of nicotine-d_7. The mass transitions, retention times and LLOQs are summarized in Table 9.
The calibrations were performed using the standard addition method with weighting. For this purpose, increasing amounts of the unlabeled standard compound were spiked in water. The calibration ranges from 0.5 to 1500 pg/ml.

4.3.6 Determination of creatinine

Creatinine was determined for the option to normalize the urine samples. For this purpose, all urine samples including Part A and B were analyzed resulting in 978 samples.

4.3.6.1 Chemicals

Creatinine ($\geq$ 98 %, anhydrous) and an aqueous picric acid solution (0.9-1.1 %) was supplied by Sigma Aldrich (St. Louis, Missouri, United States of America). Sodium hydroxide solution (5 mol/l in water), hydrochloric acid (31.5 % in water) and water (Emsure grade) were obtained from Merck KGaA (Darmstadt, Germany). For sample cleanup, different solutions were prepared. These were a 1 g/l creatinine solution, a 0.1 mol/l hydrochloric acid and a 0,25 mol/l sodium hydroxide solution.

4.3.6.2 Instrument and software

The microplate reader Genios (Tecan, Krailsheim, Germany) was used for the creatinine determination. The data processing and evaluation was conducted with the software Magellan™(Tecan, Krailsheim, Germany) and Excel 2013 (Microsoft, Redmond, USA).

4.3.6.3 Method description

Sample preparation and measurements were performed according to a validated SOP and DFG method [197]. Briefly, the urine samples were 1:51 diluted with water. Subsequently, the samples were centrifuged at 6000 rpm for 5 min at ambient temperature. All samples were measured in duplicates on 96-well plates. 50 µl sample were transferred into a well and 200 µl of a freshly prepared 1:1 solution of the sodium hydroxide solution and picric acid solution were added. The 96-well plate was closed with a lid and placed in the plate reader for incubation and measurement. After 45 minutes at 36 °C the plates were stirred for 60 s and the absorption measurement at 492 nm with 60 scans was started.

Quantification was performed with the standard addition method. Calibration was performed with a set of 8 calibrators to achieve an appropriate calibration range (2 - 70 µg/ml). Due to the lack of analyte-free matrix samples, the calibration was prepared in ultrapure water as surrogate matrix.

4.4 Statistical methods

Data evaluation was performed for all subjects and group comparisons were done for the vaping subgroups (10 W and 18 W). Note that dosimetric data based on labeled precursors obtained from vapers should not be compared to those of the smokers of the positive control group due to the completely different product designs. Urinary excretion kinetics were evaluated on an individual basis while plasma and saliva PK analysis was assessed both on an individual level as well as group-wise. The data set was too small to evaluate normal distribution, yet, it was postulated that the data is not normally distributed. Therefore, a non-parametric test (Kruskal-Wallis test) for group comparisons was applied . The described statistical tests and the data plotting was conducted with GraphPad Prism 8 (San Diego, US). Raw data were evaluated with regard to mean $\pm$ SD; median; min; max; AUC (if applicable); confidence intervals (CI 95) were calculated using GraphPad Prism 8 (San Diego, US), SPSS (IBM, Armonk, US) and Microsoft Excel 2013 (Microsoft, Redmond, US).

5 Results

5.1 Clinical Study

The clinical study was performed between 11-Nov-2016 and 18-Feb-2017 at the clinical research organization (CRO) CTC North in Hamburg, Germany. Ethical approval was received on 13-Sep-2016 according to the Helsinki declaration [184] by the ethical commission of the Medical Chamber of Hamburg (Germany).
25 healthy male subjects participated in the clinical study; five of them were experienced smokers and the other 20 experienced vapers. The 20 vapers were randomly assigned to either the high wattage group (18 W) or the low wattage group (10 W) and divided into 5 subgroups (SG 2-6). The e-vaper subgroups completed successfully Part A. One subject in SG 3 refused to participate in Part B of the study due to personal reasons. Consequently, only 19 of the 20 vapers participated in Part B. Subgroup 1 included five smoker (positive control group) who completed only the Part A of the study according to the study plan.
The participants of the study were between 21 and 56 years old (mean age 39.4). The mean age of 20 vapers was 38.0 (21 – 56) years, that of the 10 subjects vaping with 10 W in Part A 37.7 (24 – 56) years and of the 10 subjects vaping with 18 W was 38.3 (21 – 50) years. The mean age of the 5 cigarette smokers ('positive control' in Part A) was 44.6 (25 – 53) years. Mean ages between the various subgroups were not significantly different.
The vapers in the clinical study self-reported 90 (15 - 245) puffs/d resulting in a mean e-liquid consumption of 11.6 (1.5 - 50) ml/d. The cigarette smokers stated a mean daily cigarette consumption of 29 (15 - 40). In Part A (controlled vaping) of the study, 10 W vapers consumed, on average, 1.26 (range: 0.74 – 1.66) g/10 sessions. The corresponding values for the 18 W vapers were 1.48 (0.60 – 3.11) g/10 sessions. The difference was statistically not significant. In each session 10 puffs were taken resulting in 100 puffs over the day. During Part B (free vaping of the e-cigarette with labeled liquid), the average e-liquid consumption was 1.69 (0.08 - 4.65) g/d and the chosen wattage was 10.8 (5.0 - 20.0) W.
The sampling was largely carried out according to the study plan. One blood sample in SG 1 (123: smoker) could not be collected due to difficulties with the venipuncture. In the same group two urine container were interchanged (121 and 124: smoker) and an exact assignment to respective subject was not possible anymore. The two urine samples were excluded from the analysis. The biggest issue was the sputum collection. The sputum collection was executed non-induced. During the SG2 it was decided to limit the generation of sputum to two due to the exhaustion of the subjects. Therefore only a little number (instead of 216) of sputum samples were collected. Overall the smoker had less difficulties with collecting sputum samples than the vaper. Another problem of the collection of non-induced sputum is the differentiation between sputum and saliva. Thus the matrix sputum was not analyzed during this thesis. A detailed summary of deviation is shown in Appendix C in Table 30.

Adverse effects
The smokers did not report any adverse event which were related to the study procedure. Adverse events were reported for four subjects in SG 2. One subject (104; 10 W) fainted during the blood

draw, which most likely was an effect of the blood drawing process rather than the vaping. The subject had to lay down for the following blood draws. No further adverse events were reported for this subject. The second subject (105; 18 W) vomited during the fourth vaping session. He was asked if he would like to abort the study which he denied. Furthermore, he was encouraged to change his rather intensive vaping behavior, in order to better cope with the inhaled vapor. The study PI was informed and gave his approval to continue the study. The third subject (101; 18 W) reported heartburn after vaping during both Part A and B of the study. Another subject (103: 18 W) stated to feel pressure in the head after the first vaping session. The feeling disappeared on the second study day and did not reappear in Part B of the study. Two subjects developed a thrombophlebitis in their elbows due to the vein catheter. The effect was resolved by removing the catheter and some concomitant medication. Still, the blood draw could be continued. A summary of adverse events is provided in Appendix C Table 29.

5.2 Liquid and aerosol characterization

5.2.1 Propylene glycol, Glycerol and Nicotine

The labeled reference standards purchased from Synthèse AptoChem and the 100 % labeled liquid containing $^{13}C_3$-PG, $^{13}C_3$-G, and N-d_7 prepared in-house were quality controlled with regard to purity and amount/abstinence of unlabeled compound by means of the newly developed GC-MS method (Section 4.2.1). Isotopic purity was confirmed for all three analytes. In analogy, the composition of the e-liquid used for this study supplied by Happy Liquid was confirmed regarding its PG, G and N content. The study e-liquid containing 10 % of labeled PG, G and N, intended for use in the clinical study was quality controlled (Table 10). Analysis of the applied study e-liquid yielded 41.4, 47.0 and 1.0 % of unlabeled PG, G and nicotine, respectively. The corresponding percentages for the labeled constituents were 5.1, 5.4 and 0.1 %. Characterization of the prepared e-liquid proved the correct composition of PG, G, and N in the unlabeled as well as in the labeled form. The clinical study was performed using the same lot of custom-made liquid. Using identical e-cigarette devices and e-liquid derived from the same production lot ensured comparability throughout the entire study for all vaping subgroups.

Table 10: Mass percentages (% (w/w)) of the unlabeled and the labeled liquids as well as the final spiked liquid which was used in the clinical study. PG: propylene glycol; G: glycerol; N: nicotine.

	PG	$^{13}C_3$-PG	G	$^{13}C_3$-G	N	N-d_7
	[% (w/w)]		[% (w/w)]		[% (w/w)]	
Unlabeled e-liquid (Happy Liquid)	44.8	-	53.9	-	1.2	-
Labeled e-liquid (100 % labeled e-liquid)	-	50	-	50	-	1.3
Study e-liquid (Unlabeled e-liquid spiked with 10 % labeled e-liquid)	41.4	5.1	47.0	5.4	1.0	0.1

The same method as for the e-liquids was applied to stable-isotope labeled e-liquid aerosols. The aerosol was generated by means of a 1-channel-smoking machine at 10 W and 18 W and trapped on a Cambridge filter (CF). The measurements were conducted for the two different powers and the spiked non-filter cigarette including 8 replicates. The average consumption of e-liquid was 1.54 g per 100 puffs (10 W) and 2.72 g per 100 puffs. The e-cig aerosol obtained under the conditions described in Section 4.2 contained 4.04 mg PG, 3.30 mg G, 0.087 mg nicotine, 0.46 mg $^{13}C_3$-PG, 0.45 mg $^{13}C_3$-G, and 0.008 mg nicotine-d_7 per puff when vaped with 10 W. Corresponding values for vaping with 18 W were 4.67, 6.58, 0.138, 0.56, 0.87, 0.86 and 0.012 mg/puff. Non-filter cigarettes spiked with 13.4 mg $^{13}C_3$-PG, 13.6 mg $^{13}C_3$-G, 0.32 mg nicotine-d_7 yielded 0.13, 0.18 and 0.013 mg/puff of the labeled PG, G and nicotine, respectively. In general, higher levels of labeled PG and

G were obtained in e-liquid aerosols compared to the non-filter cigarette smoke under the applied conditions.

The e-liquid manufacturer was asked to provide an e-liquid with 50:50 PG:G including 1.2 % nicotine. The results of the analysis showed that the e-liquid met this expectations. The labeled e-liquid and the study e-liquid were internally prepared and also met requirements. All the six analytes were quantifiable in aerosol of the study e-liquid and mainstream smoke of the spiked cigarette. The obtained ratio fitted well to the desired values.

5.2.2 Carbonyls

Seven aldehydes (formaldehyde (FA), acetaldehyde (AA), acrolein (ACR), crotonaldehyde (CR), propionaldehyde (PA), acetone (AT) and methacrolein (MA)) were measured in e-cigarette aerosol and cigarette mainstream smoke as well as in the e-liquid in their labeled and unlabeled form. Due to the high background contamination of the carbonyls, especially for FA, AA and AT, all presented data are background corrected. The carbonyl concentrations was assessed in a ten-fold determination.

In cigarette mainstream smoke, all carbonyls which were expected to be formed from labeled PG and G according to Figure 3 were detected (labeled FA, AA, ACR, CR and PA). In addition, ^{13}C-labeled AT and MA were observed. Mean concentrations ranged from 6.3 ng/puff for ^{13}C-FA to 290 ng/puff for $^{13}C_3$-AT (Table 15). The corresponding unlabeled compounds were mostly obtained at much higher amounts in smoke between 74 ng/puff (FA) and 31,000 ng/puff for AA.

The observed per puff amounts in the aerosol of the EC were lower than in the cigarette smoke by several orders of magnitude both for the labeled and unlabeled carbonyls, except for FA where the difference was less pronounced (2- to 4-fold higher levels in smoke). Only ^{13}C-FA, $^{13}C_2$-AA and $^{13}C_3$-ACR were quantifiable in the aerosol, averaging 2.4 and 4.4 ng/puff, 0.23 and 0.55 ng/puff as well as 0.7 and 1.1 ng/puff for the 10 W and 18 W vaping conditions, respectively. Notably, the increase of these carbonyls in the 18 W compared to the 10 W vaping conditions was around 1.6- to 2.3-fold. ^{13}C-labeled CR, MA and PA were not detectable (below the LOD) and $^{13}C_3$-AT was below the LLOQ in EC aerosol. In analogy to the labeled carbonyls, vaping at 10 W yielded lower amounts than at 18 W for the unlabeled analytes. The following rank order was observed for unlabeled carbonyls: methacrolein (< LLOQ) < crotonaldehyde (< LLOQ at 10 W and 0.27 ng/puff at 18 W) < propionaldehyde < acetone ≈ acrolein < formaldehyde ≈ acetaldehyde (see Figure 12). A ratio (R = amount unlabeled/amount labeled) of around 10 was observed for FA and ACR in aerosol reflecting the 10 % replacement of PG and G in the e-liquid, while AA yielded a significantly higher ratio of 81 to 84 (Table 15).

Besides the calculation per puff for the sake of comparison with the mainstream smoke data, the concentrations of labeled FA, AA, and ACR were also determined based on the e-liquid consumption as ng/g e-liquid. This normalization takes into account the variability in aerosol generation due to the differing wattage applied. Thus, concentrations on a per gram e-liquid basis should be better suited to compare aerosol amounts between studies regardless of the puffing regime applied [94].

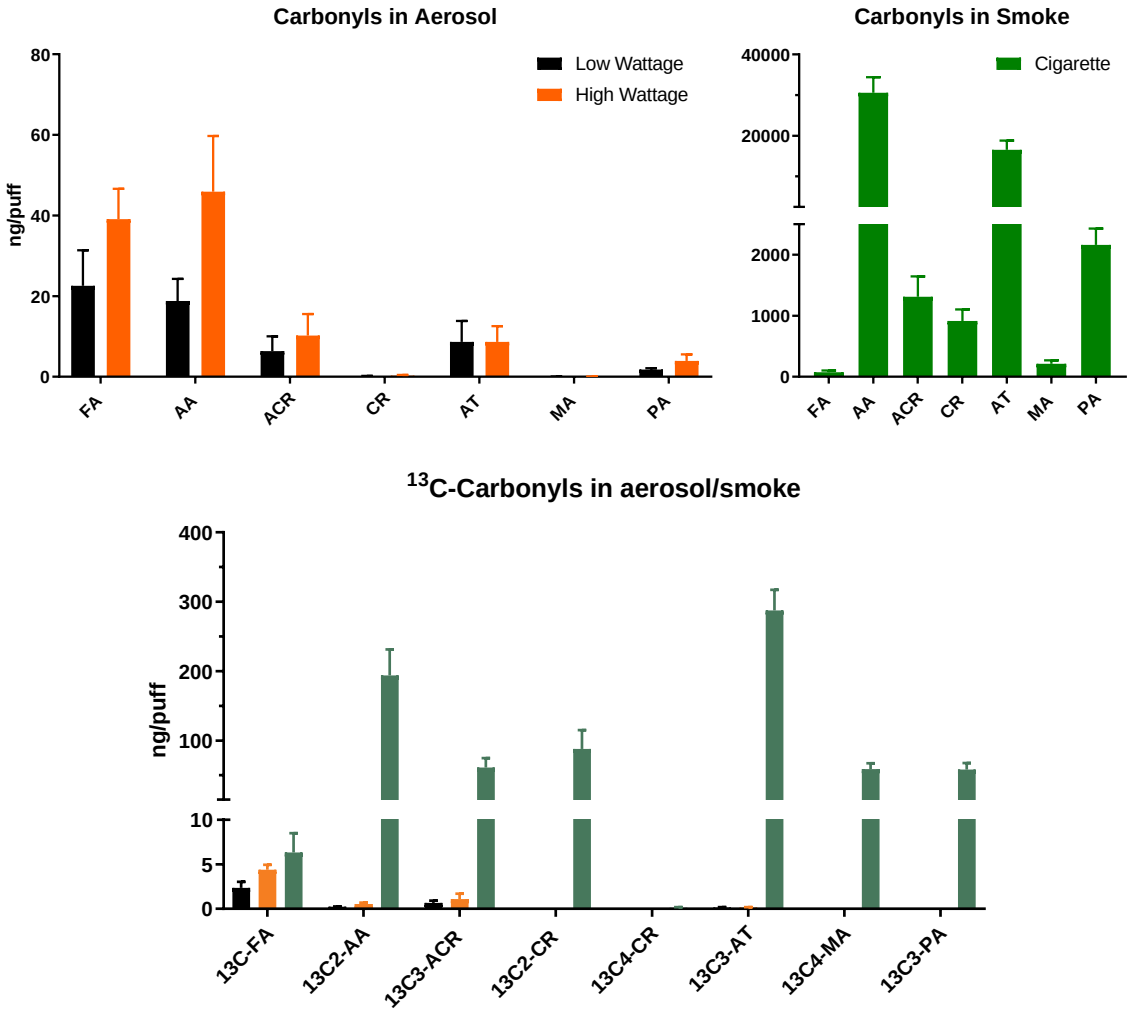


Figure 12: Comparison of the unlabeled (top) and the labeled (bottom) carbonyl amounts per puff in e-cigarette aerosol (aerosol) and main stream smoke (smoke) (black = 10 W; orange = 18 W) and smoke (green); background corrected. The bar graphs illustrate the mean with the 95 % confidence interval. Formaldehyde (FA); acetaldehyde (AA); acrolein (ACR); crotonaldehyde (CR), propionaldehyde (PA); acetone (AT) and methacrolein (MA).

There were no significant differences in the concentrations based on e-liquid consumption between the two power settings with highest concentrations found for labeled FA (150 and 162 ng/g e-liquid) followed by labeled ACR (80 and 80 ng/g e-liquid) and AA (15 and 20 ng/g e-liquid).
Additionally, the carbonyl concentration of the study liquid was assessed in a ten-fold determination. Unlabeled FA and AA were detected in amounts of 100 ng/g liquid and 265 ng/g liquid. The other five carbonyls were not detected (< LOD). Small amounts of labeled AA were measured in the study liquid. But labeled FA were not found in the liquid.
The formation of FA, AA and ACR during e-cigarette use can be attributed to PG and G because these three analytes were quantifiable in their labeled form. The spiked cigarette work as positive control since the formation of the seven labeled carbonyls were observed.

5.2.3 Tobacco specific nitrosamines

In order to assess the thermal decomposition of nicotine to tobacco specific nitrosamines, NNN, NNK, NAB and NAT were analysed in smoke, aerosol and the e-liquid. Labeled and unlabeled TSNA were not detected in e-liquid and the aerosol of the e-liquid. In the mainstream smoke, only the unlabeled TSNAs were detected (NAB: 6.34 $\pm$ 1.56 ng/puff; NAT: 34.80 $\pm$ 5.19 ng/puff; NNK: 28.29 $\pm$ 5.75 ng/puff; NNN: 62.47 $\pm$ 15.25 ng/puff). The measured amounts fit well with previous publications [139, 198, 199].

5.3 Nicotine and its metabolites

5.3.1 Urinary metabolites

The urinary metabolites include nicotine (N) and its ten metabolites (cotinine (Cot); 3'-trans-hydroxy-cotinine (OH-Cot); glucuronides of nicotine (N-Gluc), cotinine (Cot-Gluc) and 3-OH-Cot (OH-Cot-Gluc); nicotine-N-oxide (NNO); cotinine-N-oxide (CNO); nornicotine (NorN); norcotinine (Nor-Cot); 4-hydroxy-(3-pyridyl)-butanoic acid (HyPyBut)). All analytes were quantifiable in their unlabeled and labeled form except for CNO-d_7. The measured metabolites cover almost of 95 % of the nicotine uptake [74].
The ratio between the labeled and unlabeled metabolites exceeded mainly the factor ten due to the long half-life of the metabolites with exception of nicotine. Smokers excreted, on average, the lowest amounts of labeled metabolites and the highest amounts of unlabeled metabolites of the 3 subgroups. A significant difference between the two vaper groups was not observed. The labeled N, Cot, NorN, NorCot, N-gluc, NNO, HyPyBut were within the observation period fully excreted ($<$ LLOQ after 61 h). The other three cotinine metabolites were still quantifiable.
Additionally, the total nicotine equivalents (TNE) were calculated for the three subgroups. TNE is the molar sum of nicotine and its 10 major metabolites expressed as nicotine mass (see equation 8) .

$$\text{TNE [µg]} = \sum \frac{\text{excreted mass (metabolite)}}{\text{M (metabolite)}} * \text{M (nicotine)} + \text{ excreted mass (nicotine)} \qquad (8)$$

TNE = total nicotine equivalents
M = molar mass

The time pattern for the excretion of TNE is shown in Figure 13. The shown data encompassed the urine samples of Part A of the study (72 h). The amounts of labeled and unlabeled TNE excreted in urine show the following rank order for the 3 subgroups: labeled TNE: cigarette smokers $<$ 10 W vapers $\approx$ 18 W vapers; unlabeled TNE: 10 W vapers $\approx$ 18 W vapers $<$ cigarette smokers; Figure 13. The elimination half-lives of the urinary nicotine metabolites (12 – 20 h, [200] prevent that 10-times of the labeled TNE amounts reach the actually measured amounts of unlabeled TNE (Figure 13). The total amount of labeled TNE after the 10 vaping/smoking sessions amounted to 760, 810 and 470 µg for the 10 W, 18 W vapers and cigarette smokers, respectively, corresponding to 7.6, 8.1 and 13.2 mg nicotine (labeled + unlabeled) taken up in the experimental vaping/smoking sessions. The urinary excretion of labeled TNE was not complete after 72 h (62 h after the last vaping/smoking session) due to the above mentioned analytes.

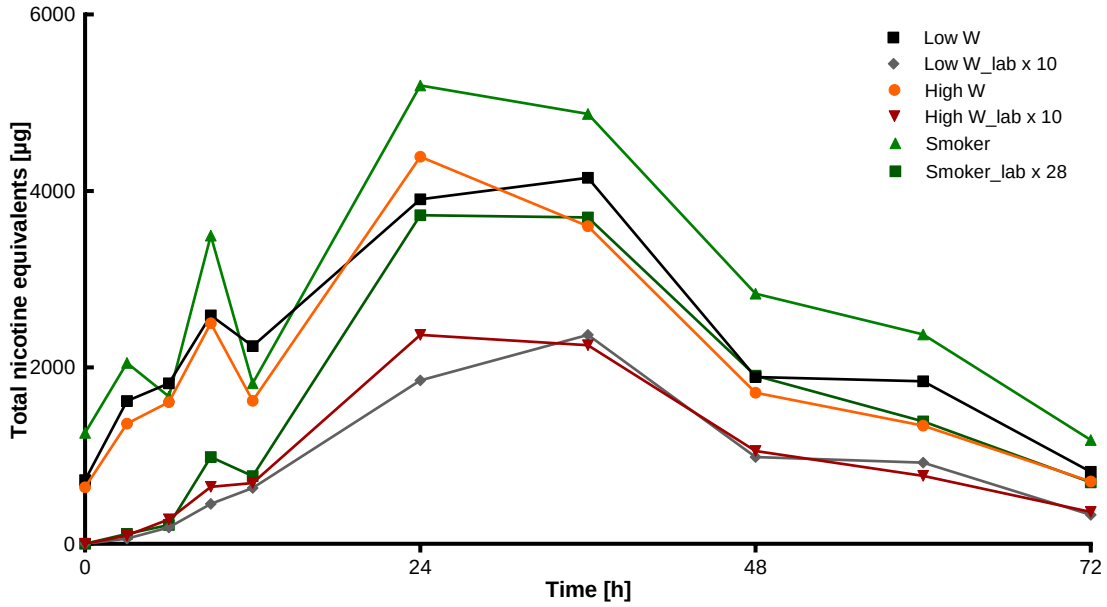


Figure 13: Time pattern for the excretion of labeled and unlabeled total nicotine equivalents (TNE) in two vaping groups (10W (black/grey) and 18 W (orange/red)) and one smoking subgroup (green) during Part A of the clinical study. The labeled TNEs are multiplied by 10 for the vapers (according to the spiking) and 28 for the smokers (deduced form nicotine plasma concentrations). Error bars are not shown for a better clarity.

5.3.2 Nicotine and metabolites in plasma and saliva

Nicotine, its major metabolite cotinine and trans-3'-hydroxycotinine were determined in plasma and saliva (Figures 14, 15). Mean nicotine plasma concentrations (both labeled and unlabeled) for all 3 subgroups (vapers with 10 W, vapers with 18 W, cigarette smokers) are shown in Figure 14 (a). After each vaping/smoking session, a peak in the nicotine level was observed resulting in a saw-tooth-shaped curve with ten maxima. A longer decline was observed between the fourth and the fifth vaping (smoking) session which was caused by the lunch break of approximately two hours. Range of maximum concentrations of 0.91 – 3.35 ng/ml (mean: 1.39 ng/ml) for nicotine-d_7 was obtained in the vapers of the 10 W group. In the 18 W group, the corresponding values were moderately, but not significantly higher (range: 0.40 – 5.47, mean: 1.90 ng/ml). The area under curve (AUC over the time period of 72 h of Part A) for labeled/unlabeled nicotine in plasma was calculated to amount to 0.73/7.17, 0.89/8.56, 0.45/12.45 ng/ml * h for low wattage, high wattage vapers and cigarette smokers, respectively. In the vapers, plasma levels of unlabeled nicotine were 10 times higher than that of the labeled alkaloid, reflecting the ratio in the e-liquid. In the smokers, the ratio of unlabeled to labeled nicotine in plasma was about 28. Higher variations in the high wattage compared to the low wattage group was observed. Smokers showed slightly lower concentrations of labeled nicotine, whereas levels of unlabeled nicotine were higher as compared to the vapers. The highest mean nicotine plasma levels (unlabeled + labeled) were observed after the last (10th) vaping session and amounted to 16.5 and 19.8 ng/ml for the 10 and 18 W vapers, respectively. The 5 smokers reached the mean maximum of 31.5 ng/ml after the 8th cigarette. Plasma levels of labeled nicotine decreased

within 48 h (36 h after the last vaping/smoking session) below the LOD of the analytical method. Plasma levels of labeled and unlabeled cotinine for all three subgroups are shown in Figure 14 (b). Vaping/smoking session-dependent peaks for cotinine, if any, were only observed for the labeled metabolite. Maximum cotinine plasma concentrations for all subgroups were reached about 2 h after the last vaping/smoking session. Labeled plasma cotinine increased from zero (prior to the first vaping/smoking session) to maximum levels of 3.5, 4.4 and 2.5 ng/ml in 10 W vapers, 18 W vapers and cigarette smokers, respectively. Cotinine (labeled and unlabeled) was still measurable in the last blood sample drawn (61 h after the last session). AUC for labeled cotinine amounted 3.46, 4.92 and 3.02 ng/ml * h for low wattage, high wattage vapers and cigarette smokers, respectively.

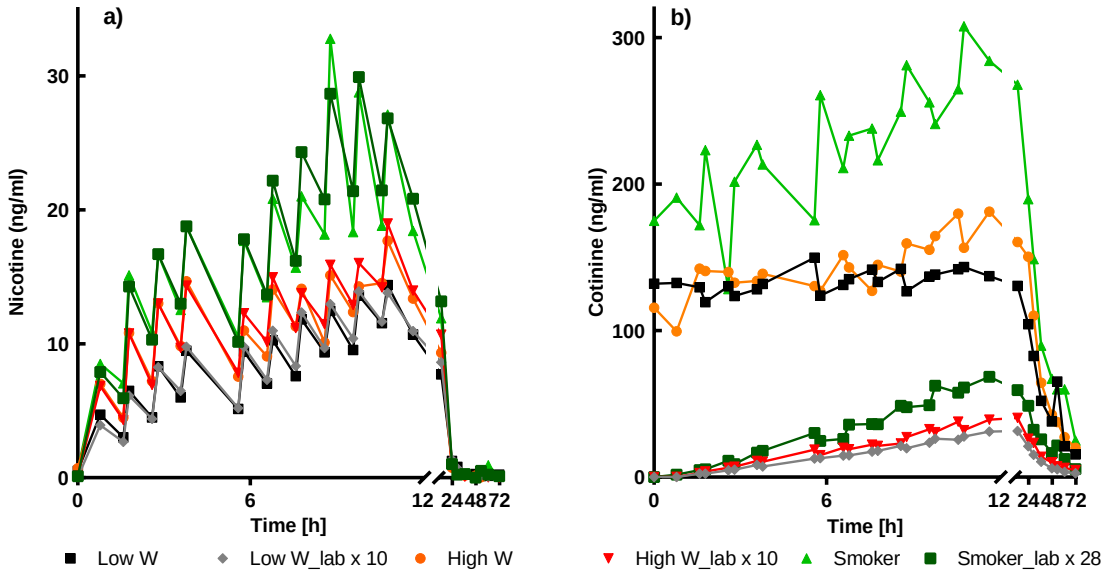


Figure 14: Comparison of the nicotine uptake of vapers (black/grey = 10 W (Low W); orange/red = 18 W (High W)) and smokers (light green/dark green) in plasma. The labeled (lab = d_7-labeled analyte) concentrations are multiplied by 10 for the vapers (reflecting the spiked amount) and 28 for the smokers. Figure a shows the results for nicotine and Figure b illustrates the results for cotinine over 72 hours. Error bars are not shown for a better clarity.

Cotinine and trans-3'-hydroxycotinine (3-OH-Cot) were measured as metabolites of nicotine in saliva. Time patterns for salivary concentrations of cotinine (labeled and unlabeled) were very similar to those in plasma (Figure 15). Prior the first vaping session unlabeled cotinine started with relatively high background. Whereas the labeled cotinine start at 0.0 ng/ml. The time pattern for salivary cotinine resembles that of plasma cotinine, however, no distinct peaks for labeled cotinine in saliva of vapers were observed. Mean maximum levels of labeled cotinine at the end of Day 1 amounted to 1.8, 2.4 and 1.2 ng/mL for 10 W vapers, 18 W vapers and cigarette smokers, respectively. Corresponding AUCs for labeled cotinine in saliva were 2.4, 3.1, and 1.7 ng/ml * h. Differences between the two vaping groups for salivary cotinine data were not significant.

OH-Cot concentrations in saliva show similar time patterns for Part A (Figure 3) as the corresponding cotinine levels. Since OH-Cot is secondary metabolite of nicotine (cotinine is a primary metabolite), peak levels of labeled OH-Cot appeared almost 12 h later than those for labeled cotinine (Figure

15b). The highest concentrations of labeled 3-OH-Cot in saliva occurred significantly later than that for labeled cotinine (14 h after the last vaping/smoking session). The maximum concentrations of the labeled OH-Cot were 3.00 ± 0.69 ng/ml, 2.96 ± 0.76 and 4.46 ± 0.70 for the 10 W vaper, 18 W and smoker, respectively. AUC values for labeled OH-Cot in saliva amounted to 0.43, 0.42 and 0.31 ng/ml * h for 10 W, 18 W vapers and cigarette smokers. The differences for the two vaping groups were not statistically significant. In the sample subset of one subject, nor cotinine neither OH-cotinine were measurable in plasma and saliva.

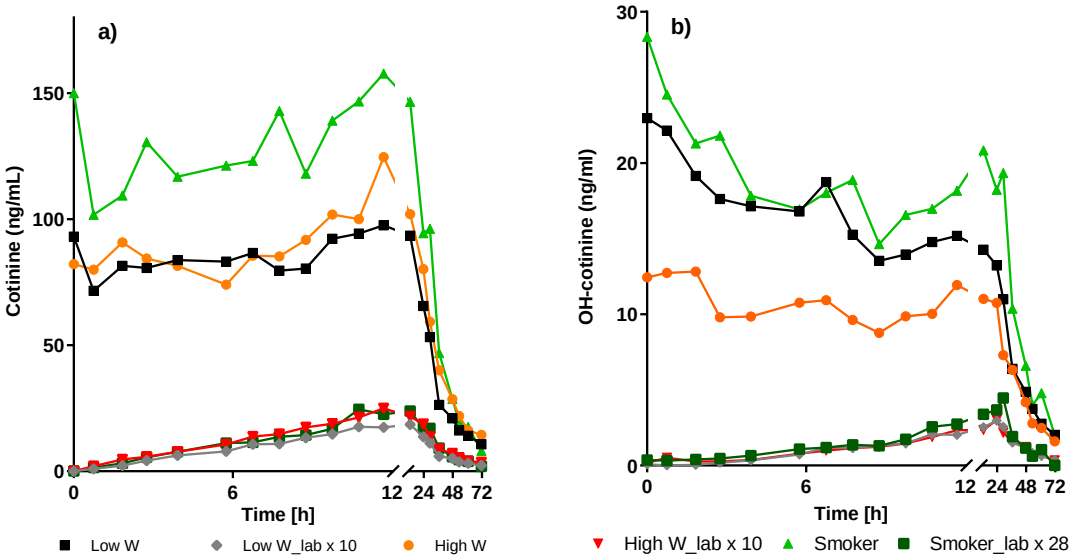


Figure 15: Salivary concentrations of labeled and unlabeled cotinine (a) and OH-Cot (b) in two vaping (black/grey = 10 W; orange/red = 18 W) and one smoking (light green/dark green) subgroup during Part A of the clinical study. Note that the labeled compounds were multiplied by 10 and 28, respectively. (Low W = 10 W group, High W = 18 W group, lab = d_7-labeled analyte). Error bars are not shown for a better clarity.

In the vapers, plasma levels of unlabeled nicotine were 10 times higher than that of the labeled alkaloid, reflecting the ratio in the e-liquid. In the smokers, the ratio of unlabeled to labeled nicotine in plasma was about 28. Apart from nicotine, all the other unlabeled metabolites were measurable at the start of the clinical study. Using the nicotine-d_7 as precursor allowed the unequivocal assignment of the metabolites to the study e-liquid. The nicotine uptake during the clinical was quiet similar for both power settings (10 W and 18 W).

5.4 Propylene glycol and Glycerol

Labeled and unlabeled PG and G levels were measured in 3 different bodyfluids (plasma, saliva, urine) of the 3 different groups. The shown data is already multiplied with the factors defined by the nicotine uptake (vaper: 10 and smoker: 28) representing the spiking levels.
G did not show a vaping-session dependent increase neither in plasma of vapers nor smokers. Plasma levels of the unlabeled G stayed at a level of 30 – 40 µg/ml in all three subgroups, independent of the vaping/smoking sessions (Figure 16, right). Labeled G in plasma were at or below the LOD of the method in all 3 subgroups.
Plasma PG levels in vapers showed defined peaks after each session for both labeled and unlabeled PG. Corresponding levels for smokers were much lower and were less distinct after smoking a cigarette. PG yielded labeled maximum concentration (c_{max}) of 0.20 µg/ml (10 W), 0.21 µg/ml (18 W) and 0.075 µg/ml (smoker). Maximum concentrations of PG were reached after the last (10th) vaping session (t_{max} = 10.8 h). In case of unlabeled PG, a background level between 0.5 and 1 µg/ml was observed while no background was detected for $^{13}C_3$-labeled PG. After 24 h (equivalent to a time period of 13 hours after the last vaping session), plasma levels of labeled PG were below the LOD of the analytical method. AUC for labeled plasma PG over 24 h were calculated to amount to 0.078, 0.091 and 0.031 µg/mL * h in 10 W vapers, 18 W vapers and cigarette smokers, respectively. Ratios of unlabeled plasma PG (10 W: 13.7; 18 W: 14.4; smoker: not applicable) to labeled PG were significantly higher than 10, due to the background of unlabeled PG caused by sources other than the e-cigs. However, no differences with reference to the applied power were found.

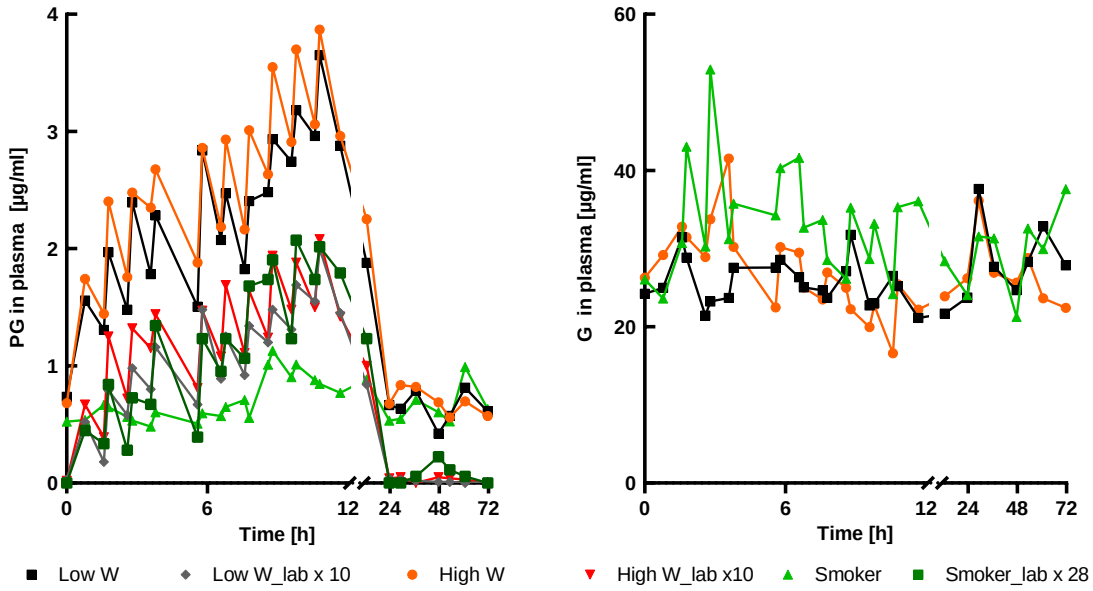


Figure 16: Plasma concentrations of propylene glycol (PG) and glycerol (G) over 72 hours in vapers (black/grey = 10 W; orange/red = 18 W) and smokers (light green/green). The $^{13}C_3$-labeled concentrations are multiplied by 10 (vapers) and 28 (smokers) based on the results of plasma nicotine concentrations. For G are only the unlabeled concentrations are shown because the $^{13}C_3$-labeled were not quantifiable. Error bars are not shown for a better clarity.

PG and G were also determined in saliva . No vaping/smoking session-dependent pattern (meaning no increase after each session was observed) for both analytes (neither labeled nor unlabeled) was found (Figure 17). The labeled concentrations were increased from zero (prior first session) to 25 to 40 µg/ml after the first session. For the next 10 h the $^{13}C_3$-PG varies between 8 and 40 µg/ml with no association to the consumed product. Vapers, in general, were observed to have higher unlabeled PG in saliva compared to smokers on Day 1, when the vaping/smoking sessions took place. The labeled PG in the smoker showed fairly the same time curve as for the vaper. The unlabeled G is unvarying at a concentration between 0.5 and 3 µg/ml over the whole study time except for the high power group. They show at Day 1 an increase after the first session comparable to the PG time curve. Overall, high variability is observed in the saliva data in all 3 subgroups.

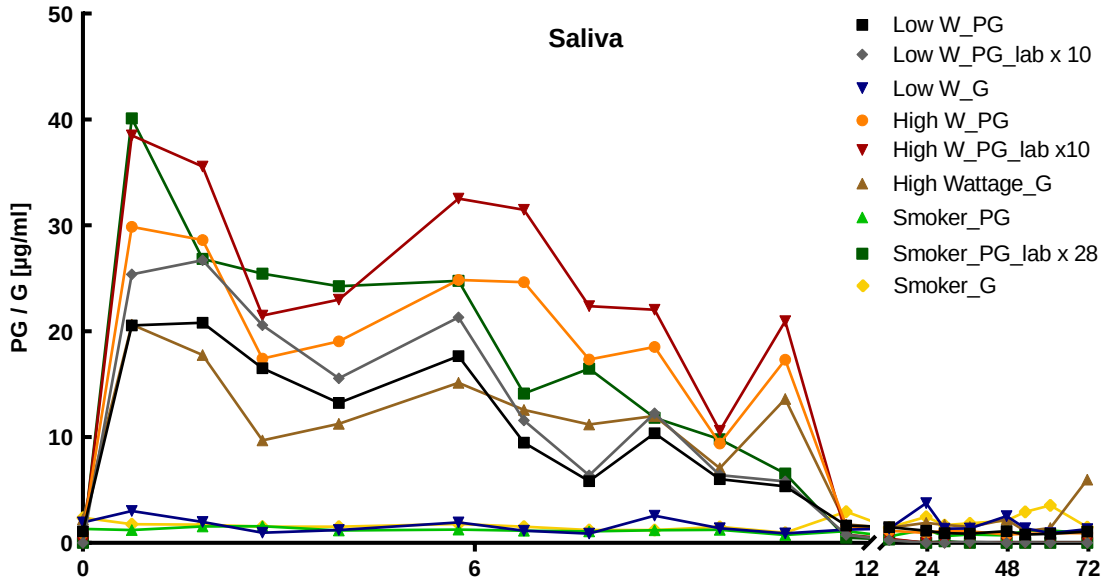


Figure 17: Salivary levels of labeled and unlabeled PG and G in two vaping (10 and 18 W) and one smoking subgroup. Note that the labeled results already multiplied by ten due to the 10% replacement of the e-liquid.(Low W = 10 W group, High W = 18 W group, lab = $^{13}C_3$-labeled analyte). Error bars are not shown for a better clarity.

Urinary excretion kinetics of PG and G for all 3 subgroups are shown in Figure 18. The peak excretion of PG (labeled and unlabeled) in all groups was reached shortly after the last vaping/smoking session. The excretion occurring from the study device was completed about 36 h after the last exposure since the levels of $^{13}C_3$-PG were no longer detectable. The unlabeled PG concentration goes back to the initial concentration during the same time period. It is noteworthy that no $^{13}C_3$-G was detectable in any of the subgroups. Unlabeled G in urine showed no relationship to the vaping/smoking sessions.

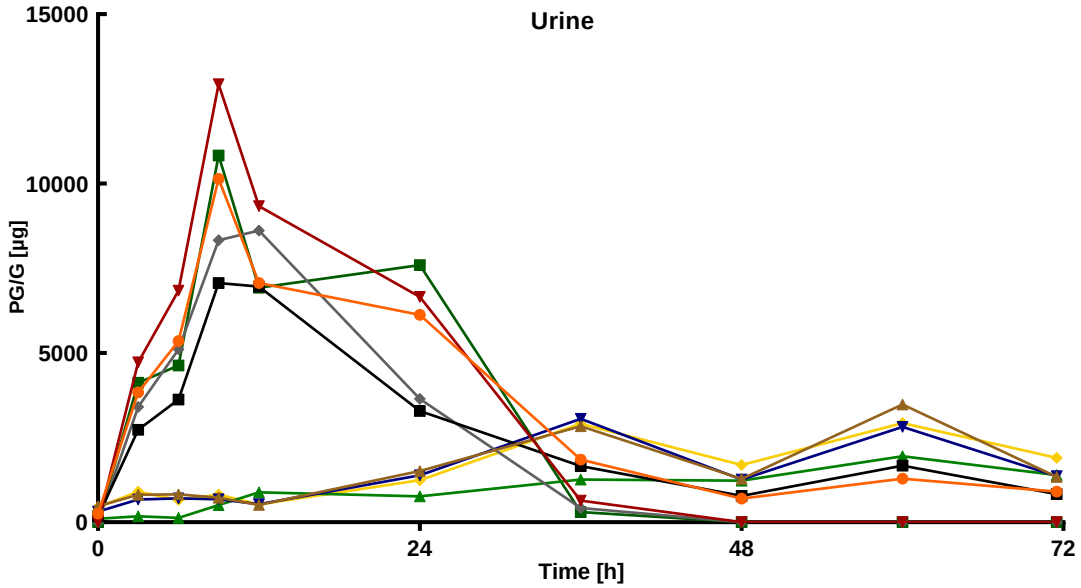


Figure 18: Urinary levels of labeled and unlabeled PG and G in two vaping (10 and 18 W) and one smoking subgroup (Part A). Note that the labeled results already multiplied by ten due to the 10% replacement of the e-liquid. (Low W = 10 W group, High W = 18 W group, lab = $^{13}C_3$-labeled analyte)

Propylene glycol is a suitable biomarker for recent e-cigarette use except for saliva. The half-life seems to be similar to nicotine. Glycerol is not a suitable biomarker because the labeled glycerol was not quantifiable in the body fluids and the unlabeled glycerol stayed at the same level over the whole observation period.

5.5 Mercapturic acids

HEMA (biomarker of ethylene oxide) and HMPMA (biomarker of crotonaldehyde) were measured together in MA I (cf. Section 4.3.3). Both unlabeled analytes were detected in the urine samples over 48 h. The excretion of HEMA was similar between the two vaping groups (Mean: 10W: 7.7 µg, 18 W: 6.8 µg). Compared to the smoker (Mean: 12.1 µg), the excreted amount was only half as large (Figure 19, left). The excreted amounts varied from 8.4 to 16.6 µg for the smoker and 3.2 to 12.6 µg for the vaper. HMPMA showed a similar behavior. The vaper excretions were in the same range (88 - 312 µg) and the smoker excreted in the same observation period twice as much (363 - 455 µg, Figure 19, right). The labeled analogues of both analytes were not detected in any of the study groups, including the positive control group.

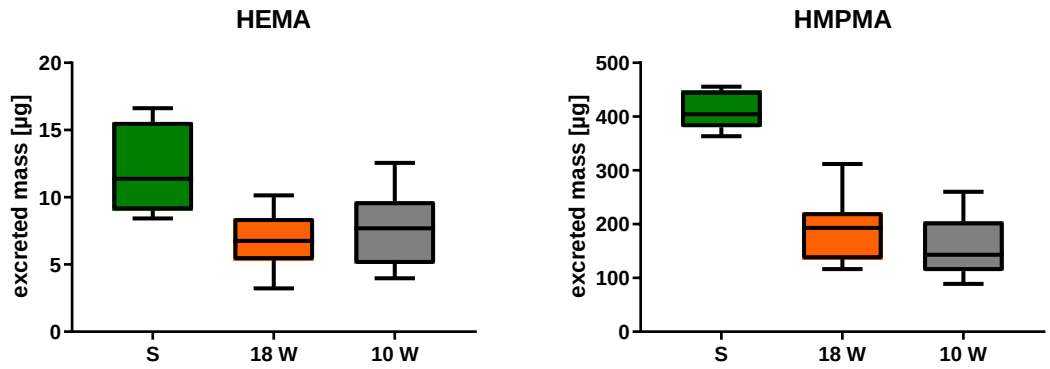


Figure 19: Boxplot diagram excreted masses of HEMA (N-Acetyl-S-(2-hydroxyethyl)-L-cysteine, biomarker of ethylene oxide) and HMPMA (N-Acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine, biomarker of crotonaldehyde) over 48 h in all three groups (vapers (10 W and 18 W) and smokers (S)). The whiskers represent the 95 % confidence interval.

The other three mercapturic acids (2-HPMA, 3-HPMA and DHPMA) were analysed using the MA II (cf. Section 4.3.3). 2-HPMA, the mercapturic acid of propylene oxide, was found in the two vaper groups in similar amounts (10 W: 20.4 - 53.4 µg, 18 W: 15.8 - 43.7 µg). The results do not indicated a power associated increase. In comparison to the smoker the excreted amounts were approximately half as large (Smoker: 26.2 - 84.6 µg). For $^{13}C_3$-2-HPMA, amounts between 2.8 and 78.1 µg excreted after 48 hours in urine were observed in the smoking group (Figure 20). In contrast, no $^{13}C_3$-2-HPMA was detectable in the urine samples of the vaping groups despite the highly sensitive determination which was applied (LLOQ of $^{13}C_3$-2-HPMA: approx. 1.25 ng/ml). The excretion was completed 24 h after the last smoking session (Figure 20 right top). The highest excretion rate were reached shortly after the last vaping session.

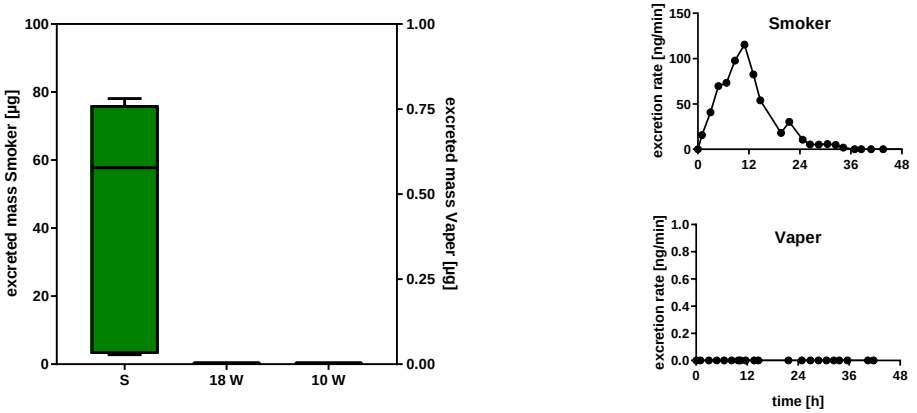


Figure 20: Boxplot diagram for excreted mass of $^{13}C_3$-2-HPMA over 48 h in all three groups (left). The whiskers represent the 95 % confidence interval. Representative excretion rates of $^{13}C_3$-2-HPMA for smokers (upper right) and vapers (lower right) over 48 h.

Similarly, 3-HPMA, as metabolic product of acrolein in urine, was detected in the same pattern as 2-HPMA but with distinct higher concentrations (Smoker: 700 - 1491 µg; 10 W: 219 - 670 µg; 18 W: 240 - 568 µg). $^{13}C_3$-3-HPMA was detected (96.2 – 197.5 µg total amount excreted after 48 hours) in the smokers. $^{13}C_3$-3-HPMA levels were lower (mostly < LLOQ) in the vaping subgroups (0.1 – 1.5 µg total amount excreted after 48 hours). Nevertheless, vaping-dependent excretion kinetics were found for $^{13}C_3$-3-HPMA in most of the vapers (Figure 21, right, bottom). Yet, it has to be noted that the concentration ranges found in the vapers were far lower compared to the smokers. Urinary levels of $^{13}C_3$-3-HPMA were about 300 times lower in vapers compared to smokers. There was not a power associated increase of 2-HPMA formation in the two vaper groups observed (Figure 21, left)

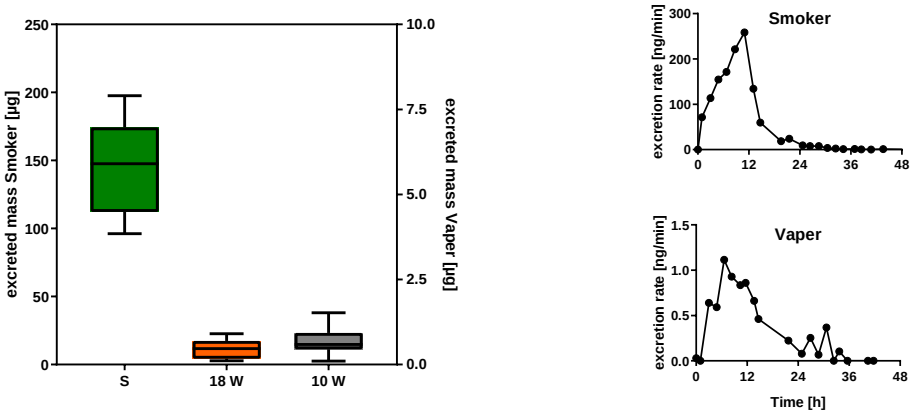


Figure 21: Boxplot diagram for excreted mass of $^{13}C_3$-3-HPMA over 48 h in all three groups (left). The whiskers represent the 95 % confidence interval. Representative excretion rates of $^{13}C_3$-3-HPMA for smokers (upper right) and vapers (lower right) over 48 h.

Finally, the highest labeled MA concentrations in e-cigarette vapers were found for $^{13}C_3$-DHPMA,

the MA of glycidol (Figure 22, left). It was found that 2.4 – 8.7 µg total amount of labeled DHPMA was excreted within 48 hours in vapers, indicating a moderate formation and subsequent metabolism of glycidol specifically attributed to vaping. In comparison, $^{13}C_3$-DHPMA formation during combustion of spiked CC was significantly higher, as can be concluded from urinary $^{13}C_3$-DHPMA concentrations in the smoking subgroup (10.8 – 31.7 µg total amount excreted within 48 hours). As for labeled 3-HPMA, a session-dependent excretion kinetic in urine was observed for $^{13}C_3$-DHPMA in both vapers and smokers (Figure 22, right). Average amounts of $^{13}C_3$-DHPMA excreted by the vaping groups with 10 and 18 W were similar. Hence, there is not a power dependent increase observed.

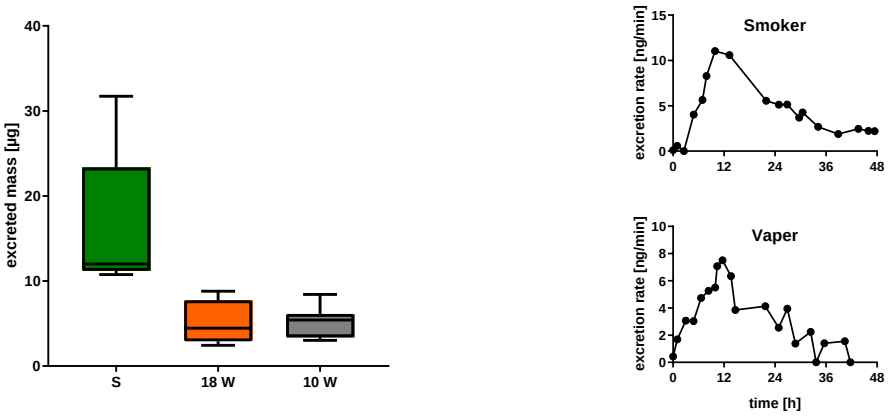


Figure 22: Boxplot diagram for excreted mass of $^{13}C_3$-DHPMA over 48 h in all three groups (left). The whiskers represent the 95 % confidence interval. Representative excretion rates of $^{13}C_3$-DHPMA for smokers (upper right) and vapers (lower right) over 48 h. The whiskers represent the 95 % confidence interval.

These results show that $^{13}C_3$-acrolein ($^{13}C_3$-3-HPMA) and $^{13}C_3$-glycidol ($^{13}C_3$-DHPMA) are formed during aerosolization from $^{13}C_3$-propylene glycol and $^{13}C_3$-glycerol, respectively. In contrast the other investigated biomarkers ($^{13}C_2$-HEMA, $^{13}C_{2/4}$-HMPMA, $^{13}C_3$-2-HPMA) were not quantifiable in the vapers. $^{13}C_3$-2-HPMA (biomarker of propylene oxide) was detected in the urine samples of smokers, suggesting that propylene oxide is formed during pyrolysis from propylene glycol. $^{13}C_2$HEMA (biomarker of ethylene oxide) and $^{13}C_{2/4}$-HMPMA (biomarker of crotonaldehyde) also were not quantifiable in smokers.

5.6 Biomarker of formaldehyde and acetaldehyde

Thiazolidine-4-carboxylic acid (TCA) and thiazolidine-4-carboxyl glycine (TCG) are specific biomarkers of exposure of formaldehyde. 2-Methylthiazolidine-4-carboxylic acid (MTCA) and 2-Methylthiazolidine-4-carboxyl glycine (MTCG) are both specific biomarkers of exposure of acetaldehyde. All four biomarkers were measured in urine and plasma.
Their unlabeled forms did not show a session dependent increase for any of the subgroups in plasma (Figure 23). In addition, there was no difference in the concentrations of the unlabeled analytes between the vaping and the smoking groups. The plasma concentrations ranged from 1.1 to 6.5 ng/ml for TCA and 2.7 to 28.1 ng/ml for TCG. MTCA and MTCG showed a similar high inter-individual variation as TCG/TCA resulting in 3.08 $\pm$ 0.41 ng/ml (vaper) and 2.37 $\pm$ 1.23 ng/ml (smoker) for MTCA as well as 0.98 $\pm$ 0.21 ng/ml (vaper) and 0.77 $\pm$ 0.40 ng/ml (smoker) for MTCG. The presented data for the both vaper groups (10 W and 18 W) were combined for a better overview. The concentration of the $^{13}C_3$-labeled analogues were below the LOD of the analytical method in plasma.

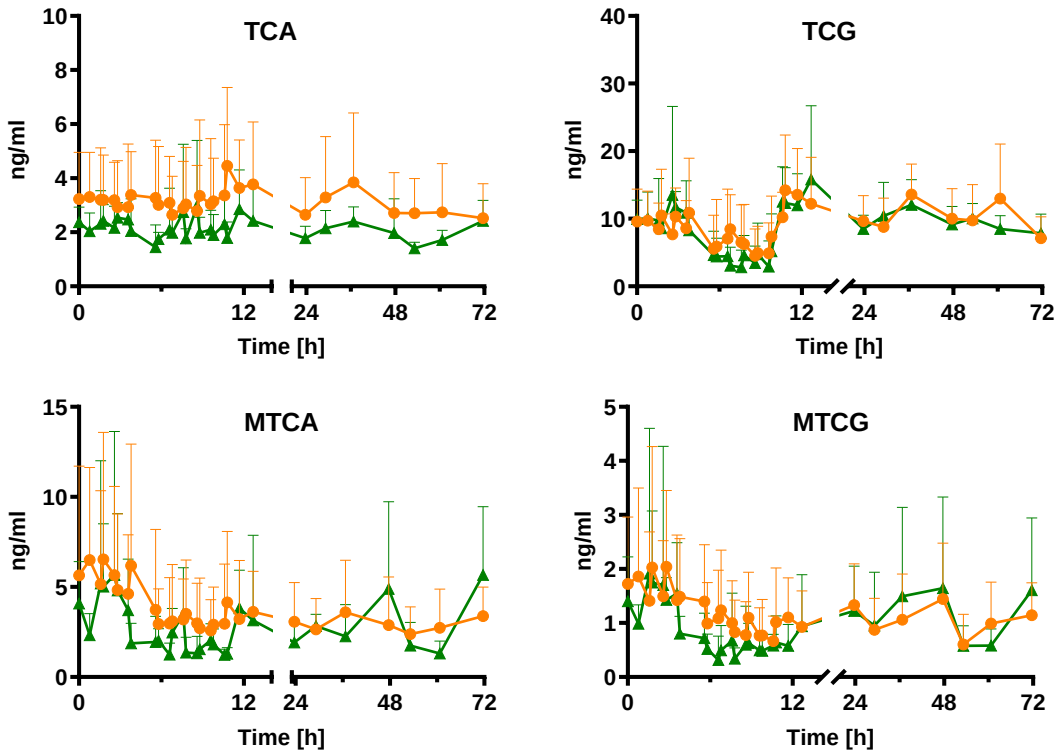


Figure 23: Plasma concentrations of Thiazolidine-4-carboxylic acid (TCA),thiazolidine-4-carboxyl glycine (TCG), 2-Methylthiazolidine-4-carboxylic acid (MTCA) and 2-Methylthiazolidine-4-carboxyl glycin in vaper (orange) and smoker (green) over 72 h. The vaping/smoking sessions took place between 1 and 12 h. This area is stretched for a better visualization. The two vaper groups were combined for a better overview.

The excreted amounts of the unlabeled biomarkers over 50 h across all groups were from 175 to 786 µg for TCA and 987 to 5012 µg for TCG, respectively. There was no difference observed between the smoker and the vaper. Significant differences were observed between smokers and vapers for the labeled TCA and TCG. Figure 24 shows a significant accumulation in smokers and a slight

accumulation in vapers. The urinary excretion of $^{13}C_3$-labeled TCA and TCG was completed 14 hours after the last cigarette which indicates a smoking session dependence. For vapers, concentrations were mostly obtained below the LLOQ . Hence, the smoking group which served as a control group clearly proved the applicability of the analytical approach in case of formaldehyde (TCA).
In contrast to TCA, no measurable excretion of labeled MTCA was observed after the vaping/smoking sessions. The three different groups showed similar trends, both for labeled and unlabeled MTCA. However, the concentration of the labeled MTCA was mostly below the LOD for all subjects. During method development it appeared that MTCA was not stable over time, neither in standard solutions nor in QC material (urine). A significant decline in MTCA concentrations was already observed within one week in urine. Therefore, the missing response to vaping/smoking for MTCA could be attributed to the lack of stability and does not necessarily reflect the absence of the formation of acetaldehyde from PG or G during vaping/smoking.

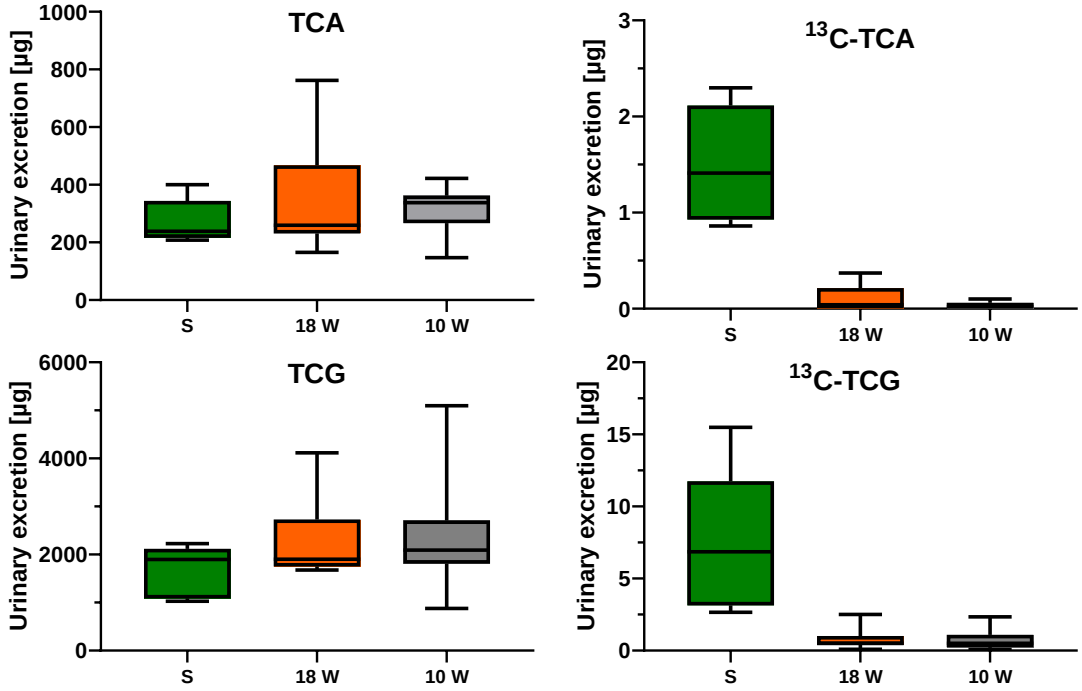


Figure 24: Box plots of the total amounts of the biomarkers excreted over 48 h after controlled vaping (10 W, 18 W)/smoking (S). Lines and boxes represent the median and the 25th/75th percentile, respectively. Error bars illustrate the 5th and 95th percentile.

Thiazolidine-4-carboxylic acid (TCA) and thiazolidine-4-carboxyl glycine (TCG) are suitable urinary biomarkers of formaldehyde since both were formed in their labeled as well as unlabeled form in smokers and vapers after exposure to the study e-liquid and the spiked cigarette. Consequently, the formation of formaldehyde from propylene glycol and glycerol through thermal decomposition was proven. The discovered amounts in the vapers are clearly lower than in smokers. 2-Methylthiazolidine-4-carboxylic acid (MTCA) and 2-Methylthiazolidine-4-carboxyl glycine (MTCG) were only detected in their unlabeled form and did not show a difference between the smoker and the vapers. The lack of labeled MTCA and MTCG in urine might be due to the discovered instability of both substances during sample storage.

5.7 Tobacco specific nitrosamines

Based on the results of the aerosol, it was not expected any TSNA formation from labeled N as a precursor. Therefore, only a small subset of urine samples was analyzed comprising the first eight collected urine voids for smokers and two urine samples for the vapers (urine void before the first vaping session and the last urine sample of Day 1 in which one would expect the highest levels according to the study design). No labeled NNAL or NNN were detected in the selected urine samples. Unlabeled TSNAs were only measurable in the urine samples of smokers, as expected due to the fact that the precursors (NNK and NNN) are formed during tobacco production (curing) and are transferred to mainstream smoke.

These results strongly suggest that nicotine in e-liquid is not a precursor for TSNAs during vaping. Furthermore, the results with the smokers of N-spiked cigarettes suggest that nicotine in tobacco is also not a precursor for NNK and NNN in smoke.

5.8 Part B

Nineteen of the 20 vapers who participated in Part A also took part in Part B. The subjects collected the saliva samples in the morning at home and stored them in a cooling bag. Another saliva sample was collected at the clinical site in the afternoon. The urine samples were collected be the subjects themselves over the whole day and stored in cooling bag. The plasma sample was collected in the afternoon. In this different matrices, nicotine and its 10 metabolites, propylene glycol, glycerol, mercapturic acids and the biomarkers of formaldehyde and acetaldehyde were analyzed.
During Part B (ad libitum vaping of the e-cigarette with labeled liquid), the average daily e-liquid consumption amounted to 1.81 (0.08 – 4.54), 1.69 (0.19 – 4.65) and 1.58 (0.09 – 4.31) g on Day 1, 2 and 3, respectively. Use pattern was evaluated by means of the subjects' diaries for Part B (Appendix B). Mean puff number per day was 66.7 – 111.1 puffs in 24 hours. Mean wattage applied was 10.8 W which is slightly higher than the 10 W applied in the low wattage group during Part A. The maximum wattage setting in the free part was 20 W which was above the conditions for high wattage group in Part A (18 W). Hence, the wattage conditions specified in the controlled Part A reflected the range preferred by the subjects during free use rather well.

Propylene glycol and glycerol

In Part B, the subjects were asked to collect in the morning on their own saliva and urine samples. In the afternoon at the clinical site saliva and urine samples and additionally plasma samples were collected. The time patterns shown in Figure 25 represent the PG time curve under free vaping conditions. Note that the levels for the labeled metabolites were multiplied by 10, so that the orange curves should approach the blue curves during the course of Part B of the study, provided that the subjects used exclusively the e-cigarette with the spiked e-liquid (ratio unlabeled/labeled = 10). There is not a background contamination of the $^{13}C_3$-PG at the first time point in the plasma samples. At following 3 days the $^{13}C_3$-PG remains quiet stable at a concentration between 0.00 µg/ml and 4.1 µg/ml in plasma. The ratio between the labeled and the unlabeled results in 60.8.

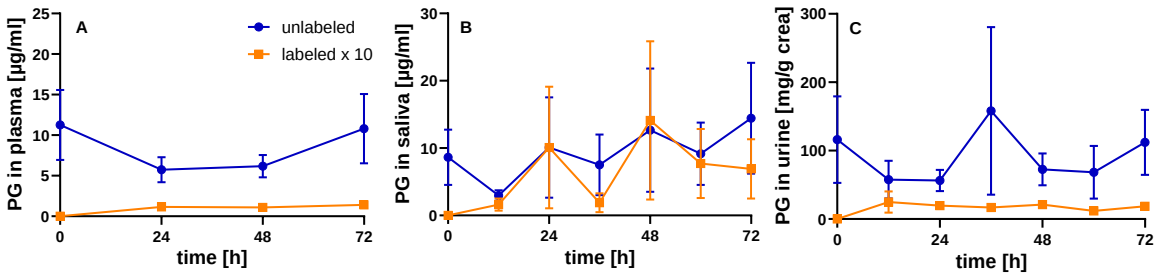


Figure 25: Time pattern of unlabeled (blue) and labeled (orange) biomarker levels for propylene glycol (PG) in plasma (A), salivary PG (B) and PG in urine (normalized to creatinine (crea)) under free vaping conditions of Part B of the clinical study. Note that the levels for the labeled metabolites were multiplied by 10, so that the orange curves should approach the blue curves during the course of Part B of the study, provided that the subjects used exclusively the e-cigarette with the spiked e-liquid (ratio unlabeled/labeled = 10) and that there were also no other sources for the uptake of PG.

In contrast the PG concentration in saliva shows a high inter- and intra-individuality which is rep-

resented be the high error bars. In time pattern of saliva (Figure 25 B) a clear vaping dependent increase can be observed. In the afternoon (24 h, 48 h and 72h), the $^{13}C_3$-PG concentration fits well to the PG concentration which can be deduced to e-cigarette consumption immediately before the sample collection. A similar behavior was observed in Part A. This indicates again that PG in saliva might not be a suitable biomarker.

The average urinary $^{13}C_3$-PG increased from 0.00 mg/g crea (before receiving the test e-cigarette) to 2.48 mg/g crea within the first 24 h. On the following days the average urinary $^{13}C_3$-PG decrease to 1.19 mg/g crea even so the subject where allowed the use the test device. The single values show also for some subject that they did not use the test device because their $^{13}C_3$-PG is 0.00 mg/g crea over all the three days.

Nicotine and its metabolites

The labeled nicotine plasma concentrations allow the calculation of a vaping-derived total plasma nicotine level of 9.0 ng/ml (Appendix D Table 32). Vaping-derived concentration is calculated from the measured labeled concentration multiplied by 10. In contrast, the mean unlabeled plasma nicotine levels reached a concentration of 14.5 ng/ml on Day 3 (Appendix D Table 32), resulting in difference between the labeled and unlabeled nicotine of 5.5 ng/ml. The ratio between labeled and unlabeled nicotine is 16.1 which is clearly higher than in Part A.

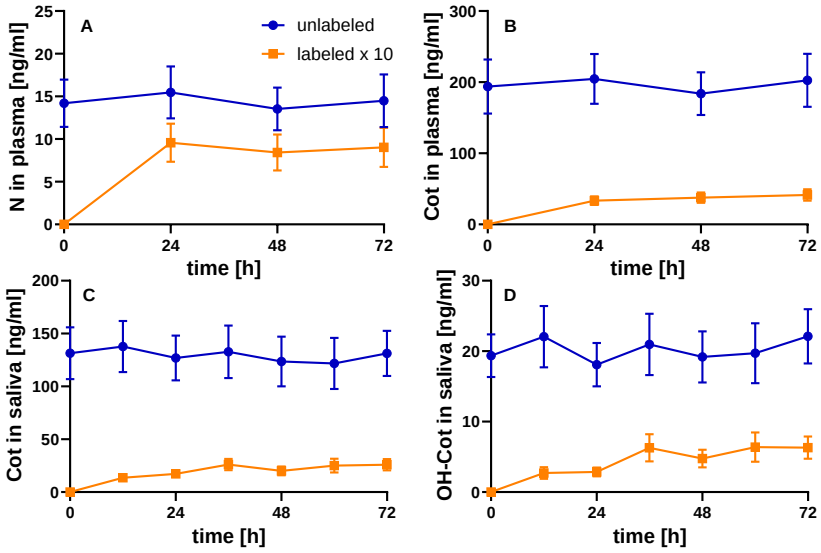


Figure 26: Time pattern of unlabeled (blue) and labeled (orange) biomarker levels for nicotine (N) in plasma (A), cotinine (Cot) in plasma (B) and saliva (C) as well as hydroxy-cotinine (OH-Cot) in saliva (D) under free vaping conditions of Part B of the clinical study. Note that the levels for the labeled metabolites were multiplied by 10, so that the orange curves should approach the blue curves during the course of Part B of the study, provided that the subjects used exclusively the e-cigarette with the spiked e-liquid (ratio unlabeled/labeled = 10).

Plasma cotinine concentrations attributable to use of the experimental e-cig in Part B (= level of labeled cotinine x 10) was 41.6 ng/ml on Day 3 compared to 202.7 ng/ml of unlabeled plasma (Fig-

ure 26). Total plasma cotinine levels (labeled + unlabeled) at the end of Part B was 206.8 ng/ml. Concentration of salivary cotinine at the end of Part B were 25 ng/mL (due to use of the experimental e-cig) and 130 ng/mL (total unlabeled cotinine) (Figure 26), thus confirming the ratios observed for plasma cotinine. Salivary OH-Cot behaved similar as cotinine.
Assuming a creatinine excretion rate of 1.5 g/d, it can be estimated that 7.5 mg/d TNE were excreted on Day 3 of Part B due to the subjects' e-cig consumption (Appendix D Table 32), which is very close to the nicotine uptake of the vapers with 10 W during the 10 experimental vaping sessions of Part A. The excretion of unlabeled TNE on Day 3 of Part B can be estimated to amount to 15 mg/d.

Thermal decomposition products

For the thermal decomposition products (MA, TSNA, MTCA/TCA), the spot urine samples did not show any trends or differences in-between vaping groups concerning the investigated biomarkers in Part B. As for Part A, labeled $^{13}C_3$-3-HPMA and $^{13}C_3$-DHPMA were found in all subjects whereas $^{13}C_3$-2-HPMA, $^{13}C_3$-HEMA, and $^{13}C_{2/4}$-HMPMA were not observed. The unlabeled MTCA and TCA showed mainly higher values in Part B than in Part A. The labeled ^{13}C-TCA and $^{13}C_2$-MTCA were mostly below the LLOQ of the analytical method (Appendix A Table 25 and 26).

6 Discussion

The applicability of the labeled e-liquid ingredients PG, G and nicotine was investigated in a clinical study with 20 experienced male vapers. In Part A of the study, subjects vaped an e-cigarette equipped with an e-liquid with 10 % of the unlabeled PG, G and nicotine replaced with ^{13}C-PG, ^{13}C-G, and d_7-nicotine, respectively, under controlled conditions. Vaping took place on Day 1 of Part A in 10 sessions, each comprising 10 puffs with a frequency of 2/min. Blood, saliva, urine and sputum samples were collected prior, during and after the vaping day until the morning of Day 4 (72 h after start of controlled vaping). As a (positive) control, 5 male smoked 10 non-filter cigarettes each, spiked with a mixture of labeled PG, G and nicotine parallel to the 10 vaping sessions.

6.1 Test products

Propylene glycol (PG) and glycerol (G) are the main ingredients in the e-liquid. In the cigarette is usually low amounts of PG and G which are used are humectant. For establishing the cigarettes as positive control in the clinical study, they needed to be spiked with a similar amount of $^{13}C_3$-PG and $^{13}C_3$-G as the consumed e-liquid per session (10 puffs). The cigarette was spiked with the labeled main ingredients over the whole length of the tobacco rod using a syringe (see Section 4.1.1). Different cigarette types were tested for this purpose. Filtered cigarette showed a very low recovery of the labeled compounds which was not exceptionally explainable with thermal decomposition. Therefore, non-filter cigarette were tested. They showed much better recoveries of the labeled ingredients. This indicates that most of the labeled compounds are absorbed by the filter of the cigarette. Additionally, many experiments were conducted with different amounts of labeled e-liquid spiked to the non-filtered cigarette. The starting point was 6.5 mg $^{13}C_3$-PG, 6.5 mg $^{13}C_3$-G and 0.16 mg N. To increase the sensitivity, the amount was doubled and quadrupled. Interestingly, the increase did not show an increase in the main stream concentration of the cigarette smoke, except for nicotine. The missing increase in labeled PG/G when using twice as much stable-isotope spike in non-filter cigarettes might be a hint for thermal decomposition of PG and G during cigarette smoking. In contrast, the concentration of N-d_7 showed an increase. However the increase was not proportional to the increase in the amount spiked to the non-filter cigarettes. N levels in smoke of the double-spiked cigarettes were comparable to the amounts found in the aerosol of the e-cigarettes used at high wattage conditions (18 W). Thus, the higher spiking levels were used for the non-filtered cigarettes in the clinical study. Hence, in the clinical study non-filtered cigarette was spiked with 13.4 mg $^{13}C_3$-PG, 13.6 mg $^{13}C_3$-G and 0.32 mg N (see Table 14).

The composition of the aerosol was also investigated prior to the clinical study. The average consumption of e-liquid was 1.54 g per 100 puffs (10 W) and 2.72 g per 100 puffs. The consumption of the 10 W conditions go with the average amount consumed by the subjects in the low wattage group (1.26 $\pm$ 0.29 mg). In contrast, the consumed e-liquid in the high wattage group (1.56 ± 0.95 mg) of the clinical study was only slightly higher than in low wattage group. The inadequate comparability between the machine consumed e-liquid and the consumption within the study might be a problem of the taste of the product and the lack of familiarization with the system.

In general, higher levels of labeled PG and G were obtained in e-liquid aerosols compared to the

non-filter cigarette smoke under the applied conditions. Around 50 % of the consumed e-liquid are trapped on the filter (Table 14). Further experiments showed that approximately 20 % are lost in the apparatus and about 25 % lost in the tank system by condensation. Since it is not clear whether the system behaves similar under usual vaping condition, the results are reported for the aerosol trapped on the filter without any correction. In the validation, the recovery of the extraction was between 93 and 106 %. Therefore the extraction procedure of the method is not the limitation. The recovery of the cigarette was even lower (around 10 %) which is probably due to the thermal decomposition. This will be further shown in section 6.4.

Moreover, we observed a higher increase in G for the higher wattage condition as compared to PG in the e-cigarette aerosol (see Table 14). This is true for both the labeled and unlabeled PG and G. An explanation could be the different boiling points and vapor pressures of the two compounds (PG: 188 °C /0.11 hPa; G: 290 °C /< 0.1 Pa) and the dependency of the vapor pressure on an increase in temperature with higher wattage.

Table 14: Comparison of the aerosol and e-liquid data in concern of the recovery from the e-liquid in the aerosol. The presented data reflect the results for 100 puffs with the e-cigarette and one spiked non-filtered cigarette.

	e-liquid ingredients	expected amounts [mg]	actual amounts [mg]	recovery (actual/ expected) [% (w/w)]	ratio of the actual amount (ratio of the e-liquid) [% (w/w)]
10 W	PG	637	404	63	48.4 (41.4)
	$^{13}C_3$-PG	78.5	46.0	59	5.5 (5.1)
	G	723	330	46	39.5 (47.0)
	$^{13}C_3$-G	83.2	45.0	54	5.4 (5.4)
	Nic	15.4	8.7	56	1.0 (1.0)
	Nic-d_7	1.54	0.8	52	0.10 (0.10)
	Total	***1540***	***834.5***	***54***	***100***
18 W	PG	1126	468	42	36.4 (41.4)
	$^{13}C_3$-PG	138	56.0	41	4.4 (5.1)
	G	1278	658	51	51.2 (47.0)
	$^{13}C_3$-G	147	87	59	6.8 (5.4)
	Nic	27.2	13.8	51	1.1 (1.0)
	Nic-d_7	2.72	1.20	44	0.09 (0.10)
	Total	***2720***	***1284***	***47***	***100***
Cig	$^{13}C_3$-PG	13.4	1.04	7.8	40.2 (49.0)
	$^{13}C_3$-G	13.6	1.44	10.6	55.7 (49.7)
	Nic-d_7	0.32	0.104	32.5	4.0(1.22)
	Total	***27.32***	***2.58***	***9.5***	***100***

6.2 Uptake of nicotine by vaping

Due to its short half-life of about 2 h, concentrations of both unlabeled and labeled nicotine were virtually zero prior to the start of the first vaping/smoking sessions (Section 5.3.2 Figure 14) [201]. Peak of nicotine plasma levels were observed after each session for all three subgroups (vapers with 10 and 18 W, cigarette smokers). For the vapers, the ratio of unlabeled/labeled nicotine ($\approx$ 10:1) reflects the ratio in the e-liquid. Vapers with 18 W showed, on average, consistently higher labeled and unlabeled plasma nicotine concentrations than vapers with 10 W. Total (sum of unlabeled and labeled) peak nicotine plasma levels in 10 W vapers (15.77 ng/ml), 18 W vapers (19.58 ng/ml) and cigarette smokers (36.03 ng/ml) were in the same range as levels reported in the literature [31, 34, 127, 202–207].

As expected, labeled plasma cotinine concentrations increased from zero (prior to the first vaping/smoking session) to maximum levels after 12 h of 3.5, 4.4 and 2.5 ng/ml in 10 W vapers, 18 W vapers and cigarette smokers, respectively (Section 5.3.2 Figure 14), corresponding to vaping-derived cotinine concentrations of 35 ng/ml (10 W vapers) and 44 ng/ml (18 W vapers). This is significantly lower than the measured unlabeled plasma cotinine levels of 221 ng/ml (10 W) and 230 ng/ml (18 W) at the end of Day 1 in Part A (Section 5.3.2 Figure 14). The reason for this observation is the long half-life of cotinine (18 h [201]). The high levels of unlabeled cotinine in plasma are caused by prior use of nicotine products. They were still elevated at the end of Day 1 of Part A.

Time patterns for salivary concentrations of cotinine (labeled and unlabeled) were very similar to those in plasma (Section 5.3.2 Figure 15). OH-Cot concentrations in saliva show similar time patterns for Part A (Section 5.3.2 Figure 15) as the corresponding cotinine levels. Since OH-Cot is a secondary metabolite of nicotine, peak levels of labeled OH-Cot appeared almost 12 h later than those for labeled cotinine.

The saliva data revealed an interesting fact in terms of metabolic polymorphism. For Subject 118 (10 W group) very low concentrations of OH-Cot (< 0.2 ng/ml) and no labeled OH-Cot was measurable in saliva samples. The observed amounts of cotinine compared to the other subjects in this group across all time points were also very low. Resulting in an extremely high Cot/OH-Cot ratio in saliva of approximately 300, while the median (min – max) level for this ratio was 5.4 (2 – 40) for the other subjects. Since CYP 2A6 is the most important enzyme for the metabolism of nicotine (Figure 27 [129]), it can be assumed that this subject might exhibit a genetic polymorphism in form of a CYP 2A6 gene deletion or mutation. The results for nicotine in plasma for Subject 118 supported the hypothesis of a CYP 2A6 polymorphism, since the plasma nicotine levels were twice as high as compared to the other subjects in the same group.

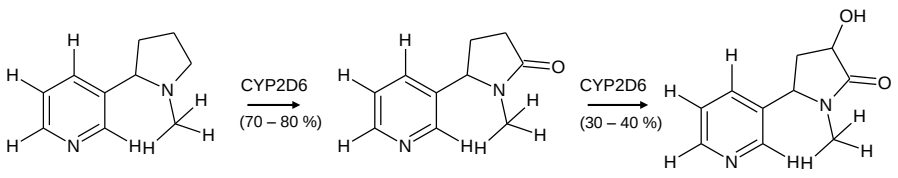


Figure 27: The role of CYP2D6 in the nicotine metabolism.

The amounts of labeled and unlabeled TNE excreted in urine show the same rank order for the three subgroups as the other nicotine biomarkers (labeled TNE: cigarette smokers < 10 W vapers ≈ 18 W vapers; unlabeled TNE: 10 W vapers ≈ 18 W vapers < cigarette smokers; Section 5.3.1 Figure 13). The amount of total TNE derived from the labeled TNE in urine (by multiplying with 10 and 28 respectively) are lower than the actually measured levels. The reason is that even after 84 h of abstinence from other nicotine products the wash-out of nicotine metabolites from the use of these products was not complete due to the long elimination half-lives of the various urinary nicotine metabolites (12 – 20 h) [200]. The total amount of labeled TNE after the 10 vaping/smoking sessions amounted to 860, 930 and 480 µg for the 10 W, 18 W vapers and cigarette smokers, respectively, corresponding to 8.6, 9.3 and 13.44 mg nicotine (labeled + unlabeled) taken up from the experimental vaping/smoking sessions. With these data, it can be estimated that the nicotine uptake per puff amounted to 86 µg (10 W vapers), 93 µg (18 W vapers) and 134 µg (cigarette smokers). This is in rather good agreement with other reports in the literature [100, 208].
Vapers consumed on average 1.26 (10 W) and 1.56 g of e-liquid during the 10 vaping sessions in Part A. This corresponds to the release of 15.17 (10 W) and 17.76 (18 W) mg nicotine by vaping. The applied method represent almost 95 % of the internal nicotine dose [74] in urine. Therefore, the 10 W vapers absorbed 53 % and the 18 W vapers 50 % of the nicotine released by the e-cig. Since there is no evidence for substantial thermal decomposition of nicotine during vaping, it can be assumed that about 50 % of the non-absorbed nicotine are lost through mouth spill and exhalation.

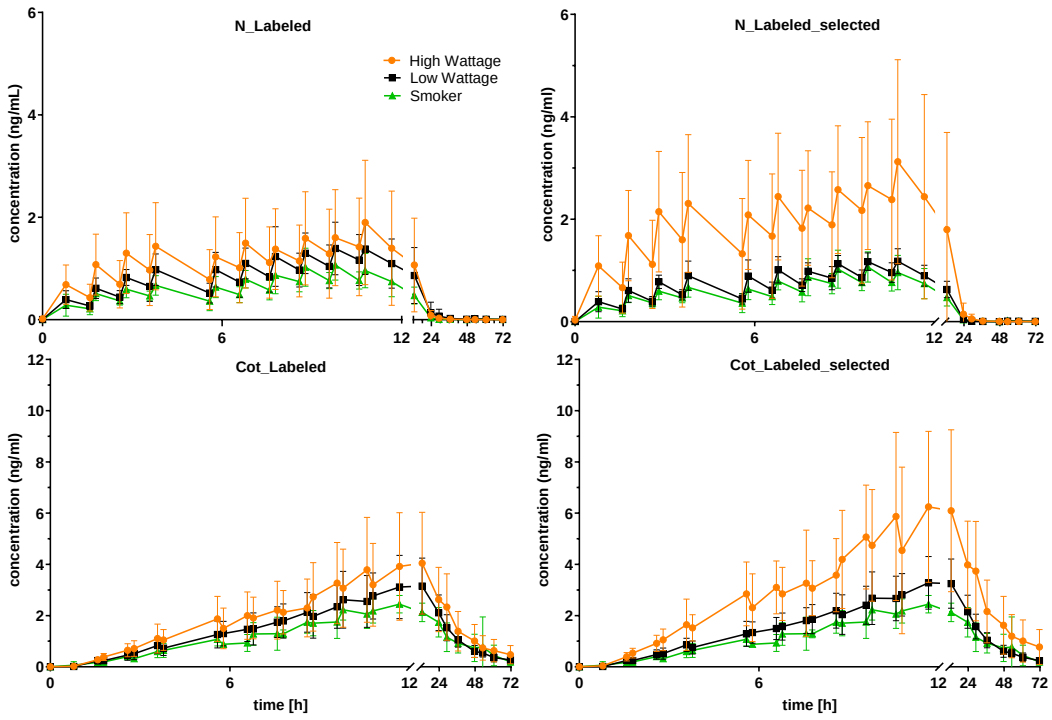


Figure 28: Plasma concentration of d_7-nicotine and d_7-cotinine. The graphs on the left side include all the subjects of the high wattage group (18 W). The graphs on the right side include only 5 vapers who did not report any trouble with the device concerning the taste, temperature etc.

There is quite a large variation in the nicotine uptake results obtained from 20 vapers and 5 smokers (SD and ranges in Appendix D Table 31), even under controlled conditions (Part A). A major contribution to this variation certainly comes from the individual puffing and inhalation behavior of the subjects. Additionally, five subjects from the high wattage (18 W) group reported problems using the product under the given conditions. When excluding those five subjects, the difference between both vaping groups became more obvious (Figure 28). Nevertheless, all subjects were included into the data evaluation for all the measured metabolites.
Apparently, the uptake of N-d_7 increased with the wattage applied during vaping. In general, the results suggest that nicotine uptake in vapers increase with the wattage appointed. It is also important to note that the plasma profiles (t_{max} and c_{max}) cannot be used for product characterization with reference to other e-vapor products as this was beyond the scope of the current project and a different study design in terms of sample collection times and product use would be required.

Labeled TSNAs were measured nor in vapers neither in smokers. Only unlabeled TSNAs were measurable in the urine samples of smokers, as expected due to the fact that the precursors (NNK and NNN) are formed during tobacco production (curing) and are transferred to mainstream smoke. These results strongly suggest that nicotine in e-liquid is not a precursor for TSNAs during vaping. Furthermore, the results with the smokers of N-spiked cigarettes suggest that nicotine in tobacco is also not a precursor for NNK and NNN in smoke.

6.3 Uptake of propylene glycol and glycerol by vaping

The time pattern for plasma PG (labeled and unlabeled) for vapers during Part A of the study resembled that for plasma nicotine for vapers. An exception being the fact that there were significant background levels at time point zero (0.5 -1 µg/ml) attributable to other sources for PG than the e-liquid. However, no differences with reference to the applied wattage were found. This would have been expected due to the aerosol data which show the factor 1.8 between the two different vaping groups. A reason for the similar time patterns in both groups might be the different vaping behavior due to the taste or nicotine concentration. The differing vaping behavior within the high wattage group was reflected in the high intra-group variations.
Smokers' PG levels were significantly lower compared to that of the vapers although the uptake of N was comparable between smokers and vapers. The time pattern observed for plasma PG suggest that PG has a half-life close to that of nicotine ($\approx$ 2 h), which is in agreement the with the literature [209, 210].
Salivary PG (both labeled and unlabeled) showed no association with the vaping/smoking sessions on Day 1 of Part A (Figure 17) suggesting that these levels originate from residual external PG in the oral cavity, rather than from systemic PG. Since this happened despite the request to the subjects to extensively flush the oral cavity with water immediately before saliva collection, it has to be noted that saliva is not a suitable biological matrix for measuring unmetabolized (parent) compounds as biomarkers of exposure through the mouth (ingestion or inhalation).
Labeled and unlabeled excretion of PG in urine showed a steep increase with the vaping session and a rapid decrease on the following day to background levels after 36 h (Figure 18). Time curves for unlabeled and 10 x labeled PG concentrations were very close together, indicating that other sources for PG (e.g. diet) were of minor importance during Part A. For the cigarette smokers, a similar time pattern was only observed for labeled PG.
Total labeled PG excreted in urine in Part A amounted to 2.94 and 4.16 mg for 10 W and 18 W vapers, respectively. These data indicate that in low wattage vapers (10 W) 29.4 mg and in high wattage vapers (18 W) 41.6 mg $^{13}C_3$-PG were excreted during the 10 experimental vaping sessions, which corresponded to per puff PG uptake of 294 and 416 µg, respectively. Vapers with 10 W consumed 1.26 g e-liquid in Part A, corresponding to an amount of 622 mg PG. Respective values for vapers with 18 W were 1.56 ml e-liquid and 771 mg PG. From the urinary PG results, it can be deduced that in human vapers only about 5 % of the generated PG from the e-cig appear as unchanged PG in urine. Two major factors might be responsible for the significantly lower recovery rate of unchanged PG compared to nicotine (recovered as TNE: 50 %): (i) PG is extensively metabolized to lactic acid, pyruvic acid and also carbon dioxide [211] (see Figure 29), presently it is not known, which percentage of the dose appears as unchanged PG in urine; (ii) absorption of PG through the buccal mucosa and the lung alveoli might be less effective than that for nicotine.
Propylene glycol can be oxidized to lactic acid via one of two pathways, depending on whether the glycol is phosphorylated [55, 212]. In in-vitro studies with rat liver, the free glycol was successively oxidized to lactaldehyde, methylglyoxal (pyuvaldehyde), and lactic acid (see pathway 1, Figure 29) [55, 213], while the phosphorylated glycol followed the pathway of acetyl phos-

phate, lactaldehyde phosphate, lactyl phosphate, and lactic acid ([214]; see pathway 2, Figure 29). Lactate is subsequently converted to pyruvate, which enters the citric acid cycle and/or the gluconeogenesis pathway [214, 215]. 1,2-Propanediol oxidation is not restricted to lactate or pyruvate formation. The corresponding deoxyaldehyde, as well as propionaldehyde, lactaldehyde, phospholipids, formate, and acetate can be formed [214]; forming glucose and carbon dioxide as metabolic end product.

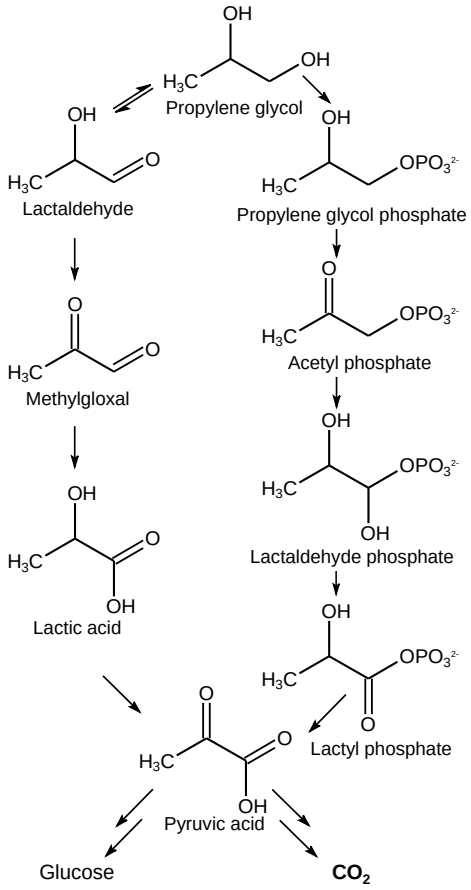


Figure 29: Metabolism of propylene glycol according to Miller et al. [55] and Rudney et al. [212]

These different metabolic products of propylene glycol are also formed by other xenobiotics as well as endogenous substances. Therefore their background concentration is relatively high. Even so, $^{13}C_3$-labeled PG was used in the study, there are too many possible biomarkers to measure them due to the lack of stability and sensitivity of the analytical methods.

In contrast to PG and N, no vaping-dependent time pattern was found for G in urine or plasma for neither the unlabeled nor the labeled analyte (Section 5.4, Figure 16 - 18). Unlabeled G already showed high basal levels of approximately 20 – 50 µg/ml in plasma (Figure 16). The maximum uptake of G using the study e-cigarette was estimated to lead to an increase of plasma levels of around 3 - 6 µg/ml based on the results for PG in plasma taking into account that PG and G are present in equal amounts in the e-liquid and in similar concentrations in the aerosol (cf. Table 14). Thus, the uptake was much lower compared to the basal level of G and, therefore, no increase in plasma levels was to be observed. Labeled $^{13}C_3$-G was not detected in plasma or urine at all. The most plausible explanation for this observation is that the amounts taken up by vaping and smoking were too small in comparison to the large physiological body pool of G and that the metabolism of G is too fast [216] to detect labeled G for example in urine (metabolic scheme in Figure 30).

Glycerol is metabolized via the glycolytic pathway after it has been converted in the liver to glycerol-3-phosphate. Glycerol-3-phosphate is then oxidized to yield dihydroxyacetone phosphate, which is isomerized to glyceraldehyde-3-phosphate, eventually yielding pyruvic acid. Pyruvic acid follows two primary routes of metabolism. Under aerobic conditions, it is converted to acetyl coenzyme A and enters the citric acid cycle. Under anaerobic conditions, primarily in muscles as a result of strenuous physical activity, pyruvic acid is reduced by lactic dehydrogenase to lactic acid. Lactic acid diffuses through muscle tissue and is transported to the liver in the bloodstream. In the liver, it is converted to glucose by gluconeogenesis. Lactic acid can also be further catabolized in the lactic acid cycle (also known as the Cori cycle).

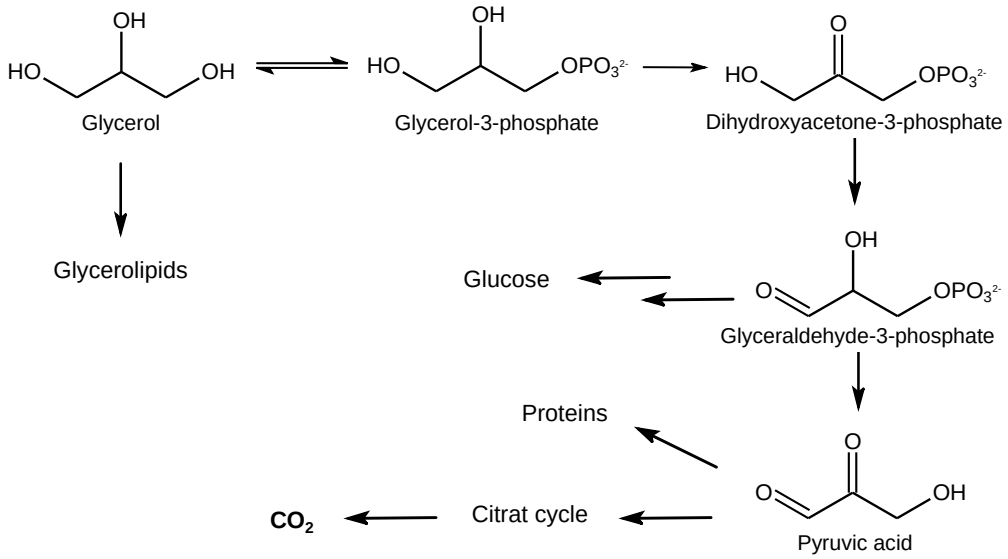


Figure 30: Metabolism of glycerol according to Hellerud, C. et al. [217]

The findings of G levels in plasma and urine are in agreement with a report in the literature showing that oral intake of G below 0.05 g/kg BW (equivalent to 4 g/ 80 kg person) had no impact on the common physiological G levels in plasma and urine [63].
Salivary G addresses the same problem as for PG. The measured amounts could be a result of the exogenous contamination through the improper rinsing of the mouth. The results clearly show that G in body fluids (either labeled or unlabeled) is not suitable as a biomarker of exposure to this e-liquid ingredient. However, labeled G is a useful precursor to investigate the uptake of thermal decomposition products such as acrolein and other aldehydes.

6.4 Thermal decomposition products and their corresponding biomarkers

The presented data unequivocally confirm the formation of formaldehyde (FA), acetaldehyde (AA), and acrolein (ACR) from the precursors PG and G in e-liquid during vaping, which is in line with previous studies [25, 91, 105, 218]. Geiss et al. who used a second-generation vaporizer device similar to the EC used in this study, found comparable levels of FA, AA, and ACR, [218] while most studies reported higher aldehyde concentrations which may result from the different device characteristics and varying puffing regimes used throughout the studies as discussed in detail by Farsalinos et al. [94]. Interestingly, the higher carbonyl concentrations per puff at 18 W (Section 5.2.2) are clearly related to increased aerosol generation as can be deduced from the fact that the amounts of carbonyls per mass consumed e-liquid were found to be identical for both wattages. This observation indicates that, at least under the vaping conditions we applied, increasing the power from 10 to 18 W elevated the amount of aerosol produced but not the percentage of PG and G which is converted to carbonyls.

Table 15: Mean yields (standard deviations) of unlabeled and ^{13}C-labeled carbonyls on a per puff basis in smoke and aerosol (ng/puff). Note that only 10 % of the e-liquid were replaced with ^{13}C-propylene glycol and ^{13}C-glycerol (50:50 (v/v)). (FA = formaldehyde, AA = acetaldehyde, ACR = acrolein, CR = crotonaldehyde, AT = acetone, MA = methacrolein, PA = propionaldehyde). R: Ratio between unlabeled and labeled concentrations in the aerosol, averaged for the amounts received at 10 W and 18 W.

	Mainstream smoke of cigarettes	EC Aerosol (10 W)	EC Aerosol (18 W)	R in aerosol 10 W	R in aerosol 18 W
FA	73.8 (26.1)	22.6 (8.23)	39.1 (8.40)	9.6	8.9
^{13}C-FA	96.0 (25.1)	2.36 (0.76)	4.39 (0.63)		
AA	30600 (3650)	18.8 (5.10)	45.9 (15.4)	81	84
$^{13}C_2$-AA	194 (49.5)	0.23 (0.05)	0.55 (0.16)		
ACR	1310 (316)	6.36 (3.43)	10.2 (5.98)	9.6	9.3
$^{13}C_3$-ACR	61.2 (18.2)	0.66 (0.22)	1.10 (0.60)		
CR	914 (181)	0.13 (0.09)	0.27 (0.19)	n.a.	n.a.
$^{13}C_2$-CR	88.1 (35.8)	< LOD	< LOD		
AT	16600 (2160)	8.66 (4.83)	8.66 (4.33)	n.a.	n.a.
$^{13}C_3$-AT	288 (39.3)	< LLOQ	< LLOQ		
MA	212 (55.4)	< LLOQ	< LLOQ	n.a.	n.a.
$^{13}C_4$-MA	59.1 (10.9)	< LOD	< LOD		
PA	2160 (255)	1.80 (0.32)	3.95 (1.79)	n.a.	n.a.
$^{13}C_3$-PA	58.4 (12.5)	< LOD	< LOD		

The introduction of labeled PG and G allowed the assessment of the formation of carbonyls specifically formed from these e-liquid constituents. The ratio of the unlabeled to the ^{13}C-labeled PG and G in the e-liquid of 10:1 was well reflected in the EC aerosol for FA and ACR (Table 15), indicating that both aldehydes are mainly (or almost exclusively) formed from PG and G during vaping. In

contrast, for AA a ratio of around 80 was observed, while CR, AT and PA were only detected in their unlabeled form. This suggests that other sources than PG and G contribute to a major extent to the formation of AA. Sugars and flavors were recently discussed as possible sources for carbonyl emissions in EC aerosols [219, 220]. The use of stable isotope-labeled flavoring ingredients and sugars could be a useful strategy in future studies to further evaluate their chemical fate with regard to vaping.

All seven ^{13}C-labeled aldehydes investigated in our study were found in cigarette mainstream smoke indicating that the formation of AA, CR, AT, and PA from PG and G is possible, albeit at higher temperatures than achievable under common vaping conditions (up to 350 °C [89]). Apparently, AA, AT and PA (C_2 and C_3 compounds) are formed due to decomposition of PG and G [98]. CR (4 carbon atoms) is more likely to be formed after decomposition of PG and G from AA by aldol condensation (Section 2.1.3 Figure 3) implying the need for harsher conditions – in this case higher temperatures [221].

Labeled biomarker analysis in urine allowed us to link the machine-derived aerosol data with actual human exposure values. The difference in smoke and vapor yields was clearly reflected in the biomarker levels for FA and ACR since the respective labeled biomarkers ^{13}C-TCA, ^{13}C-TCG and $^{13}C_3$-3-HPMA were detected at much higher levels in smokers. Hence, the approach using stable isotope-labeled precursors was capable of detecting exposure to FA and ACR specifically from vaping at a daily intake of approx. 1.1 to 2.2 µg based on the aerosol data and the average e-liquid consumption of 1.4 g per day. Unfortunately, the labeled biomarkers of AA exposure, MTCA and MTCG, respectively, could not be determined due to insufficient long-term stability in urine of only two months. Thus, MTCA and MTCG are not suited to assess AA exposure from smoking and vaping.

Furthermore, it was assumed that PG and G may also serve as precursors for the epoxides propylene oxide (PO) and glycidol (GLY) [98]. For methodological problems, the expected (labeled) epoxides could not be determined, neither in mainstream smoke of conventional cigarettes nor in aerosol of ECs. Analysis of the corresponding labeled biomarkers in urine revealed exposure to PO (2-HPMA) exclusively in smokers while glycidol exposure (DHPMA) was detected both in the case of smoking and vaping, at approx. 3-fold lower amounts in the EC vapers (Table 16). Thus, PO seemed to be formed from the precursors PG and G under pyrolytic (smoking) conditions only, while GLY was observed at appreciable amounts already under vaping conditions as can be concluded from the biomarker analysis. Moreover, there was no difference in biomarker levels between subjects using the EC at 10 and 18 W despite the higher per puff carbonyl yields in the aerosol at 18 W. In contrast, excretion rates of PG in urine correlated with the increased wattage. Presumably, the generally low exposure to carbonyls in this study was not sufficient to discriminate the vaper subgroups in terms of biomarker excretion rates. These findings emphasize the importance of biomarker analysis in addition to machine-derived aerosol data for a comprehensive exposure and risk assessment.

The difference in the ratio between the unlabeled and labeled biomarkers of exposure was much more pronounced (R = 110 – 6,500) compared with the ratios found for aerosol (9 – 84) (Table 16). Moreover, the estimated EC-specific uptake in vapers compared to the overall exposure in vapers and smokers demonstrated that EC use only accounted for approximately 0.4 %, 1.0 %, and 8 % of

the overall exposure to formaldehyde (TCA/TCG), acrolein (3-HPMA) and glycidol (DHPMA) in vapers while no EC specific uptake of PO (2-HPMA) and CR (HMPMA) was observed. This indicates other sources than vaping, like diet and endogenous formation, contribute primarily to the observed biomarker levels of TCA/TCG, 3-HPMA and DHPMA in exclusive vapers [222–228]. For example, vegetable oils and fats were recently identified as a major source of glycidol exposure [229].

Table 16: Mean (standard deviation) amounts excreted in urine of smokers and EC users (vapers) over 48 hours (µg) of the unlabeled and ^{13}C-labeled biomarkers of exposure. TCA/TCG metabolites of FA; MTCA/MTCG: metabolites of AA; 3-HPMA: metabolite of ACR; DHPMA: metabolite GLY; 2-HPMA: metabolite of PO; HMPMA: metabolite of CR. R: Ratio between unlabeled and labeled biomarker in urine of vapers.

	Smoker	Vaper		R in vapers	
		10 W	18 W	10 W	18 W
TCA	171.3 (16.5)	198.3 (44.7)	218.5 (67.6)	1,6	6,5
^{13}C-TCA	1.56 (0.55)	0.12 (0.14)	0.03 (0.04)		
TCG	912.8 (328.3)	2039 (1158)	1562 (579.9)	3,4	2,4
^{13}C-TCG	6.31 (2.97)	0.60 (0.58)	0.66 (0.58)		
MTCA	40.3 (20.1)	40.4 (9.0)	46.2 (16.5)	n.a.	n.a.
$^{13}C_2$-MTCA	< LOD	< LOD	< LOD		
MTCG	15.2 (6.9)	17.9 (4.2)	15.6 (4.2)	n.a.	n.a.
$^{13}C_2$-MTCG	< LOD	< LOD	< LOD		
3-HPMA	1057 (221.1)	381.7 (91.8)	368.9 (101.0)	810	1,3
$^{13}C_3$-3HPMA	143.2 (32.8)	0.47 (0.39)	0.28 (0.19)		
DHPMA	562.0 (122.6)	635.1 (141.1)	529.4 (69.6)	140	110
$^{13}C_3$-DHPMA	15.8 (7.78)	4.59 (1.35)	4.74 (2.36)		
2-HPMA	51.6 (17.2)	32.6 (6.7)	26.5 (7.0)	n.a.	n.a.
$^{13}C_3$-2-HPMA	43.2 (33.6)	< LOD	< LOD		
HMPMA	391.6 (31.1)	150.7 (49.7)	191.9 (56.8)	n.a.	n.a.
$^{13}C_{2/4}$-HMPMA	< LOD	< LOD	< LOD		

6.5 Part B

In Part B of the clinical study (free use of experimental e-cigs), the labeled nicotine plasma concentrations suggest a vaping-derived total plasma nicotine level of about 9 ng/ml, which is well within the range of plasma nicotine levels in vapers reported in the literature [31, 34, 127, 202–207]. Taking into account also the mean unlabeled plasma nicotine levels (14.5 ng/ml, Section 5.8 Figure 26) yielded a total nicotine level in plasma of 15.4 ng/ml, which is comparable with concentrations in medium cigarette smokers [230–232]. Furthermore, the data reveals that part of the nicotine absorption observed in Part B of the study originated from other sources than the experimental e-cig. 6.4 ng/ml (15.4 ng/ml - 9.0 ng/ml) representing 42 % of the total nicotine plasma level are resulting from other sources.

Plasma cotinine concentrations attributable to use of the experimental e-cig in Part B (= level of labeled cotinine x 10) was 41.5 ng/ml on Day 3 compared to 203 ng/ml of unlabeled plasma (Section 5.8 Figure 26), again indicating the additional use of other nicotine products during Part B of the study. Total plasma cotinine levels (labeled + unlabeled = 235 ng/ml) at the end of Part B were in agreement with those reported for regular cigarette smokers [232]. From the plasma cotinine results, it can be estimated that about 80 % of the nicotine uptake during Part B was related to the use of other nicotine/tobacco products. The discrepancy to the plasma nicotine-based estimate (42 %) can be explained with the short half-life of nicotine, reflecting the more recent use of the provided e-cig prior to the blood collection in the clinic.

Assuming a creatinine excretion rate of 1.5 g/d, it can be estimated that 18.8 mg PG/d in urine were attributable to vaping the experimental e-cig. From the e-liquid consumption (1.58 g/d, Appendix D 32), it can be calculated that 788 mg PG were taken in by vaping, thus 4.2 % of the PG measurable in urine caused by vaping, which is in fair agreement with results from Part A and also in accordance with clinical data.

Glycerol showed in all matrices the same behavior as in Part A. There was not a vaping-dependent association meaning that the levels were constantly over the whole time period.

According to the vaping diaries for Part B, all subjects consumed the study e-cigarette (containing labeled PG, G and N in the e-liquid) within a short time period before visiting the clinic for sample collection on each study day of Part B. Thus, plasma N and PG ratios (unlabeled-labeled) should be the most reliable endpoint to evaluate compliance. Two criteria were set in this evaluation: Firstly, a ratio of < 14 for N and < 30 for PG as higher ratios were most likely attributed to dual-use. A higher ratio was applied for PG compared to N because several exogenous sources except for e-cigarettes might increase the ratio. Secondly, non-compliance was assumed, if labeled N or PG was not detectable with simultaneous detection of the unlabeled compound > 20x LLOQ as this unequivocally proves consumption of a N- and/or PG-containing product different from the stable-isotope labeled e-liquid (almost no use of the Clinical Study e-cigarette). However, discrimination between non-compliance due to cigarette consumption or use of an alternative e-vapor product was rather difficult. Nine out of 19 subjects were identified as non-compliant according to the criteria given above. Three of the nine non-compliant subjects used the study e-cigarette only to a very low extent on at least one of the three study days. Although they reported a regular use pattern throughout all three days

in their diary. According to the rating of the study device in the diaries (Appendix C), most of the subjects were not satisfied with the taste of the e-liquid. In general, vape production of the device was stated to be 'alright' to 'satisfactory', however, most subjects reported that they were craving for their common e-cigarette and e-liquid brand. The rather negative overall rating of the study product may explain the high non-compliance rate of the participants.

6.6 Limitations

The results of this study should be considered in the context of the limitations. First, the relatively small sample size only of males limits the generalizability of the results, however this study does provide proof of the concept that stable-isotope labeled constituents in the e-liquid represent a suitable approach to evaluate exposure from e-liquid constituents. The use behavior may not totally reflect the real-world settings since the study participants did not use their own products and were in an in-clinic setting during the Part A.
However, this study has several advantages. The randomized controlled study design with the in-clinic setting allows a structured approach of controlled conditions of use limiting confounding factors and other sources of exposure. The design allows us to obtain direct evidence regarding uptake of constituents through measurement of consumed e-liquid and levels of stable isotope labeled constituents. The measurement of multiple biofluids allows for relatively complete characterization of the fate of the major constituents in the e-liquids.

Additionally, the sputum collection was rather difficult. For avoiding interference with medication of the induced sputum, the sputum was collected without inducing. At the clinical site, they reported to had good experience with the collection of non-induced sputum and provided a protocol. For the smoker the protocol was quiet fine but the vaper did not cope well with this procedure. Therefore, it was decided that the subject should try three times to collect the samples. If the sample collection was not possible, this time point will be excluded. Therefore, only half of the samples were collected. However, in the collected sample it was rather difficult to distinguish between saliva and sputum. Hence, the analysis of the sputum samples was neglected.

7 Conclusion

This is the first ever study that utilizes stable isotope ingredients to characterize the exposure of e-cig users. This approach overcomes limitations caused by uptake of these ingredients from other sources. The results show that replacing 10 % of the unlabeled with labeled chemicals (nicotine-d_7 and $^{13}C_3$-PG) is sufficient for quantifying the vaping-specific uptake of these ingredients. The labeled biomarkers nicotine, cotinine and PG in plasma, cotinine and OH-Cot in saliva, TNE (total nicotine equivalents based on nicotine + 10 major metabolites) in urine turned out to be highly suitable for assessing the vaping-derived uptake of nicotine and PG, even if other nicotine or tobacco products are used in parallel ('dual' users). Saliva, in general, is not suitable for measuring the parent compounds PG and G as internal dose markers, due to external PG and G in the oral cavity derived from vaping and other sources.

Labeled and unlabeled G in the investigated body fluids are not suitable biomarkers for the vaping-derived uptake of G, probably due to the high physiological levels of G and its fast metabolism. However, labeled G may be a useful precursor to investigate thermal decomposition products such as acrolein and other aldehydes as well as their biomarkers like mercapturic acids.

Additionally, this approach allows the unambiguous determination of the formation and exposure to various carbonyls and epoxides. They are formed by decomposition of propylene glycol and glycerol under smoking and vaping conditions. The formation of several toxicants such as crotonaldehyde, methacrolein and acetone was only observed during combustion of conventional cigarettes while propylene glycol and glycerol can also be decomposed to formaldehyde, acetaldehyde, acrolein and glycidol under common vaping conditions, although to a much lower degree compared to smoking.

These results were supported by the biomarker determination. The mercapturic acids derived from acrolein (3-HPMA) and to a much higher extent from glycidol (DHPMA) were found in their labeled form in urine of the vapers proving strong evidence for the formation of these toxicants upon vaping. This is the first study to prove the EC specific uptake of glycidol due to the degradation of propylene glycol and glycerol during vaping. Hence, these compounds may be important biomarkers of exposure with regard to the toxicological evaluation of this new product category.

TCA as a biomarker of exposure to formaldehyde showed an uptake-dependent increase in urine of smokers for its labeled form proving the formation of formaldehyde from the precursors PG and G during combustion. Results for the labeled TCA in urine of vapers suggest at most an uptake of trace amounts of formaldehyde by vaping. The increase after smoking was not observed for the unlabeled TCA due to the high background of unlabeled formaldehyde derived from various exogenous and endogenous sources.

These findings prove the suitability of the stable-isotope labeling approach, especially for the specific exposure to glycidol and formaldehyde which could not have been assessed by measurement of the unlabeled biomarkers due to the ubiquitous adsorption of glycidol and formaldehyde from various sources. However, vaping appears to be a minor source with respect to the general exposure to formaldehyde, acrolein and glycidol.

Also, a positive control group was successfully implemented in order to examine the potential formation and metabolism of pyrolysis products during combustion by inclusion of smokers of stable-

isotope spiked cigarettes. This positive control group proved the suitability of the general approach and in particular of the analytical methods applied in terms of sensitivity and selectivity.
In summary, these data will help to elucidate the formation of toxicologically relevant by-products due to vaping and to determine the e-vapor product specific uptake of the main constituents.

8 Perspectives

Several new questions arose during the evaluation of the study data and also after publication of novel reports in the e-cigarette-related research area. Clearly, the use of the stable-isotope labeling approach can be further extended with respect to the analysis of other compounds of interest. One report stated the generation of ^{13}C-benzene in aerosol from $^{13}C_3$-PG and $^{13}C_3$-G containing e-liquid [42]. Thus, the samples from the Clinical Study can be used to determine both benzene in aerosol/smoke and the benzene-specific mercapturic acid SPMA (S-phenyl-mercapturic acid) in urines. This approach could prove or disprove these findings.

Moreover, further aerosol experiments under harsher conditions (> 20 W; dry-puff) could be conducted to evaluate potential decomposition products. Several dicarbonyls like diacetyl, pentane-2,3-dione, methylglyoxal and others were recently reported in literature [233–235]. These compounds and their metabolites in labeled form could be analyzed as well as other carbonyls of interest (propanal, acetol) in urine and other body fluids.

The application of the stable-isotope labeling approach may pave the way to specific biomarkers of exposure to electronic vapor products. Stable-isotope labeling can be an elegant way to circumvent false positive results due to dual use and hence help to find specific biomarkers of exposure to EVP. Also this approach can be used for PK analysis and toxicological assessment of new e-vapor products. Moreover, the approach can be extended with regard to other e-liquid constituents like flavors of common use in e-liquids to address the absorption, metabolism and excretion of those compounds via the route of inhalation. The use of stable isotope-labeled flavoring ingredients and sugars could be a useful strategy in future studies to further evaluate their chemical fate with regard to vaping.

Additionally, a untargeted approach could be conducted with different nicotine delivery products. For this approach a the labeling of compounds is not necessary. Using a high resolution mass spectrometer the differences in the metabolic profile could be investigated between the different users of the nicotine delivery products. This approach might also reveal product-specific biomarkers for a better discrimination between the different users and for a better understanding of the metabolic fate.

9 Abstract

The use of electronic cigarettes (e-cigs) has steadily increased worldwide over the past decade. The potential implicated risks for the users, bystanders and the general population are topics of discussion in the scientific and public health communities. An important basis for risk estimation is a well founded dosimetry of the ingredients and other potential constituents absorbed by the users (vapers) and second-hand vapers. Based on experience with smokers, the application of biomarkers for assessing the internal dose is the most suitable approach for this purpose. A major problem is applying actually specific biomarkers for the chemicals of interest which are not derived from other sources. One way to circumvent this problem is to use stable isotope-labeled ingredients in the e-liquid.

Therefore, a clinical study with 20 subjects (refereed as vaper), vaping e-cigs equipped with e-liquids containing labeled propylene glycol (PG), glycerol (G) and nicotine under both controlled (Part A) and free conditions (Part B) was conducted. The vaper were additionally divided into two groups: Vapers using the e-cigarette with a power of 10 W (10 W vapers) and vapers using the e-cigarette with a power of 18 W (18 W vapers). In addition, five smokers, smoking 10 non-filter cigarettes each, spiked with labeled PG, G and nicotine, served as (positive) control during Part A. Various body fluids were collected and analysed by MS/MS. Additionally, the aerosol, smoke and the e-liquid were analysed to get the whole overview.

In this thesis, dosimetric results for PG, G and nicotine in various body fluids are reported. Furthermore, the formation of thermal decomposition products such as tobacco specific nitrosamines, carbonyls, epoxides and their biomarkers was investigated. Mean consumption of e-liquid of 10 vapers using an e-cig power of 10 W was 1.26 ml/10 sessions (range: 0.74 – 1.66 ml). E-liquid consumption was slightly, but not significantly higher for 10 vapers applying 18 W: 1.48 (0.60 – 3.11) ml/10 sessions.

Plasma nicotine levels peaked after each vaping (comprising 10 puffs) and smoking session (comprising 1 cigarette) in all 3 subgroups (vapers using an e-cig power of 10 and 18 W, smokers). The highest mean nicotine plasma levels (unlabeled + labeled) were observed after the last (10th) vaping session and amounted to 16.5 and 19.8 ng/ml for the 10 and 18 W vapers, respectively (difference not significant). The 5 smokers reached the mean maximum of 31.5 ng/ml after the 8th cigarette. In the vapers, levels of plasma unlabeled nicotine were 10 times higher than that of the labeled alkaloid, reflecting the ratio in the e-liquid. Labeled cotinine concentrations in plasma and saliva steadily increased over the 10 vaping/smoking sessions and then fell to undetectable levels after 2 days without vaping/smoking. The same pattern was observed for salivary trans-3'-hydroxycotinine with a shift of the peak level by several hours. The amount of labeled nicotine equivalents (nicotine + 10 major metabolites) in urine samples of Part A of the study was 810, 860 and 480 µg over 72 h for 10 W vapers, 18 W vapers and smokers, respectively.

Plasma propylene glycol levels in vapers showed defined peaks after each session for both labeled and unlabeled PG. Corresponding levels for smokers were much lower and were less distinct after smoking a cigarette. Levels of unlabeled G in plasma were similar throughout Part A for all three subgroups with no observable influence of vaping or smoking on plasma and urinary G levels. Concentrations of labeled G in plasma and urine were below the detection limit for all three subgroups.

The G findings are in accordance with the fact that a large pool of physiological G exists in the body which is not influenced by the uptake of G (either unlabeled or labeled) by vaping or smoking. Salivary PG and G showed no clear association with either vaping or smoking. The results suggest that some external PG and G might have gotten into the saliva, despite of extensive rinsing of the oral cavity before saliva samples were collected.

In Part B (ambulatory part), vapers were asked to use the same e-cigs (equipped with the labeled e-liquids) as in Part A, but were free in the set-up of the device and in the vaping pattern. Vaping the provided e-cig resulted in plasma nicotine and cotinine levels of 9 and 40 ng/ml. It could be estimated that total nicotine equivalents amounted 7.5 mg/d caused by the uptake of nicotine from the experimental e-cig. Almost all subjects used other nicotine products apart from the provided e-cig during Part B of the study.

Additionally, seven carbonyls (formaldehyde, acetaldehyde, acrolein, acetone, crotonaldehyde, methacrolein, propionaldehyde) were measured in the aerosol and the mainstream smoke. Corresponding biomarkers of exposure were determined in urine samples by LC-MS/MS. ^{13}C-labeled formaldehyde, acetaldehyde and acrolein were found in e-cig aerosol, while all seven labeled carbonyls were detected in smoke. The labeled biomarkers of exposure to formaldehyde (^{13}C-thiazolidine carboxylic acid and ^{13}C-N-(1,3-thiazolidine-4-carbonyl)glycine), acrolein ($^{13}C_3$-3-hydroxypropylmercapturic acid) and glycidol ($^{13}C_3$-dihydroxypropylmercapturic acid) were present in the urine of vapers indicating an e-cig use-specific exposure to these toxicants, albeit at much lower amounts compared to smokers. In general, the e-cig use-specific uptake only contributes to 0.4 and 1.0 % to the overall exposure to formaldehyde and acrolein and to 8 % with respect to glycidol. Other thermal decomposition products, like tobacco specific nitrosamines were not determined in the aerosol of the e-liquid unlike the cigarette. Therefore the biomarkers were not found in the vapers, neither labeled nor unlabeled.

In conclusion, the application of labeled e-liquid ingredients was shown to allow the accurate quantification of the absorption dose of nicotine and PG, but not of G, also when other nicotine and tobacco products are used simultaneously. Their resulting labeled thermal decomposition products were found in the smoke and accordingly in the smokers which served as positive control. Whereas the number and the amount of the thermal decomposition products are clearly lower in the aerosol and vaper, respectively. Linking data for the native (unlabeled) and the labeled biomarkers revealed vaping as a minor source of exposure to these toxicants. Thermal decomposition of propylene glycol and glycerol to other toxicants such as crotonaldehyde only occurs during combustion in cigarettes rather than during vaping.

10 Kurzzusammenfassung

Elektronische Zigaretten (E-Zigs) haben in den letzten zehn Jahren weltweit stetig an Zuspruch gewonnen. Die damit verbundenen potentiellen Risiken für die Nutzer (Vaper), die Passiv-Nutzer und die allgemeine Bevölkerung werden in der Wissenschaft und im Gesundheitswesen kontrovers diskutiert. Eine wichtige Grundlage für die Risikoabschätzung ist eine fundierte Dosimetrie der Inhaltsstoffe und anderer potenzieller Bestandteile, die von den Vapern und von Passiv-Vapern aufgenommen werden. Ausgehend von den Erfahrungen mit Rauchern ist die Verwendung von Biomarkern zur Abschätzung der internen Dosis hierfür der geeignetste Ansatz. Eines der Hauptprobleme ist spezifische Biomarker für die Substanzen aus der E-Zigarette zu finden und dabei andere Quellen auszuschließen. Eine Möglichkeit, dieses Problem zu umgehen, ist die Verwendung stabiler, isotopenmarkierter Inhaltsstoffe im E-Liquid.
Im Rahmen dieser Arbeit wurde eine klinische Studie mit 25 Probanden durchgeführt: 20 Vaper und fünf Raucher. Die Vaper wurden zusätzlich in 2 Gruppen aufgeteilt. Die eine Gruppe verwendete die E-Zigarette mit einer Leistung von 10 W (10 W Vaper) und die die andere Gruppe verwendete die gleiche E-Zigarette mit einer Leistung von 18 W (18 W Vaper). Diese Studie bestand aus zwei Teilen: Einem kontrollierten Teil (Part A) und einem freiem Teil (Part B). In dieser Studie wurde ein E-Liquid mit markiertem Propylenglykol (PG), Glycerin (G) und Nikotin versetzt. Die fünf Raucher rauchten während Part A 10 ungefilterte Zigaretten, die mit markiertem PG, G und Nikotin dotiert waren. Sie dienten als (positive) Kontrolle während Teil A. Verschiedene Körperflüssigkeiten wurden gesammelt und mittel MS/MS analysiert. Um einen vollständigen Überblick zu erlangen, wurde zusätzlich noch das Aerosol der E-Zigarette, der Hauptstromrauch der Zigarette und das E-Liquid analysiert.
In dieser Arbeit werden dosimetrische Ergebnisse für PG, G und Nikotin in verschiedenen Körperflüssigkeiten vorgestellt. Außerdem wurde die Entstehung von thermischen Zersetzungsprodukten, wie tabakspezifische Nitrosamine, Carbonyle, Epoxide und deren Biomarker untersucht. Der durchschnittliche E-Liquidverbrauch der 10 Vaper bei einer Leistung von 10 W betrug 1,26 ml/10 Sitzungen (Bereich: 0,74 - 1,66 ml). Der E-Liquidverbrauch der anderen 10 Vaper bei einer Leistung von 18 W war leicht, aber nicht signifikant höher: 1,48 (0,60 - 3,11) ml/10 Sitzungen. Der Nikotinspiegel im Plasma erreichte in allen 3 Untergruppen (Vaper mit 10 und 18 W, Raucher) nach jedem Vaping-Sitzung (mit 10 Zügen) und jeder Rauchersitzung (mit je 1 Zigarette) sein Maximum. Die höchsten mittleren Nikotin-Plasmaspiegel (unmarkiert + markiert) wurden nach der letzten (10.) Vaping-Sitzung beobachtet und betrugen 16,5 und 19,8 ng/ml für die 10 bzw. 18 W Vaper (Unterschied nicht signifikant). Die 5 Raucher erreichten nach der 8. Zigarette das mittlere Maximum von 31,5 ng/ml. Der Gehalt an unmarkiertem Nikotin im Plasma war bei den Vapern zehnmal höher als der des markierten Alkaloids, was das Verhältnis in dem E-Liquid widerspiegelt. Die Konzentrationen von markiertem Cotinin in Plasma und Speichel stiegen während der 10 Vaping-/Rauchsitzungen stetig an und fielen dann nach 2 Tagen ohne Vaping/Rauchen auf nicht nachweisbare Werte. Das gleiche Muster wurde für Trans-3'-Hydroxycotinin im Speichel mit einer Verschiebung des Spitzenwertes um mehrere Stunden beobachtet. Die Gesamtmenge an markierten Nikotinäquivalenten (Nikotin + 10 Hauptmetaboliten) in Urinproben von Teil A der Studie betrug 810, 860 und 480 µg nach 72 h für

10 W-, 18 W-Vaper und Raucher.
Die Propylenglykol-Plasmaspiegel in Vapern zeigten nach jeder Sitzung sowohl für das markierte als auch für das unmarkierte PG definierte Spitzenwerte. Die entsprechenden Werte für Raucher waren viel niedriger und waren nach dem Rauchen einer Zigarette weniger ausgeprägt. Die Spiegel von unmarkiertem G im Plasma waren in allen drei Untergruppen in Teil A ähnlich, ohne dass ein Einfluss des Vapens oder Rauchens auf die Plasma- und Urin-G-Spiegel beobachtet werden konnte. Die Konzentrationen von markiertem G im Plasma und Urin lagen bei allen drei Untergruppen unter der Nachweisgrenze. Die G-Befunde stehen im Einklang mit der Tatsache, dass im Körper ein großer Pool an physiologischem G existiert, der nicht durch die Aufnahme von G (entweder unmarkiert oder markiert) durch Vapen oder Rauchen beeinflusst wird. Speichel-PG und G zeigten keine klare Assoziation mit Vapen oder Rauchen. Die Ergebnisse deuten darauf hin, dass etwas externes PG und G in den Speichel gelangt sein könnte, trotz ausgiebiger Spülung der Mundhöhle vor der Entnahme der Speichelproben.
In Part B (ambulanter Teil) wurden die Vaper gebeten, ausschließlich die gleichen E-Zigs (ausgestattet mit den markiertem E-Liquid) wie in Part A zu verwenden. Sie durften allerdings die E-Zig ohne Beschränkung der Zuganzahl und Nutzungsfrequenz verwenden. Aus dieser freien Nutzung der E-Zig ergab sich ähnliche Plasmakonzentrationen für das markierte Nikotin bzw. Cotinin. Die umarkierten Konzentrationen sind jedoch deutlich höher. Die PG-Konzentrationen in Plasma und Urin PG stimmen mit den Konzentrationen aus Part A gut überein. Daher liegt die Schlussfolgerung nahe, dass fast alle Probanden in Part B zusätzlich noch andere Nikotinprodukte verwendet haben.
Zusätzlich wurden sieben Carbonyle (Formaldehyd, Acetaldehyd, Acrolein, Aceton, Crotonaldehyd, Methacrolein, Propionaldehyd) im Aerosol der E-Zigarette und im Hauptstromrauch der Zigarette gemessen. Entsprechende Expositionsbiomarker wurden in Urinproben mittels LC-MS/MS bestimmt. ^{13}C-markiertes Formaldehyd, Acetaldehyd und Acrolein wurden im Aerosol der E-Zigarette gefunden, während alle sieben markierten Carbonyle im Rauch nachgewiesen wurden. Die markierten Biomarker von Formaldehyd (^{13}C-Thiazolidincarbonsäure und ^{13}C-N-(1,3-Thiazolidin-4-carbonyl)glycin), Acrolein ($^{13}C_3$-3-Hydroxypropylmercapturinsäure) und Glycidol ($^{13}C_3$-3- Dihydroxypropylmercapturinsäure) waren im Urin von Vapern vorhanden, was auf eine E-Zig-spezifische Exposition gegenüber dieser Substanzen hinweist, wenn auch in wesentlich geringeren Mengen im Vergleich zu Rauchern. Im Allgemeinen trägt die anwendungsspezifische Aufnahme in der E-Zig nur zu 0,4 und 1,0 % zur Gesamtexposition gegenüber Formaldehyd und Acrolein und zu 8 % in Bezug auf Glycidol bei. Andere thermische Zersetzungsprodukte, wie tabakspezifische Nitrosamine, wurden im Aerosol der E-Liquid im Gegensatz zur Zigarette nicht bestimmt. Daher wurden die dazu gehörigen Biomarker in den Proben der Vaper nicht gefunden, weder markiert noch unmarkiert.
Zusammenfassend konnte gezeigt werden, dass die Anwendung von markierten E-Liquid-Inhaltsstoffen die genaue Quantifizierung der Absorptionsdosis von Nikotin und PG, nicht aber von G, auch bei gleichzeitigem Konsum anderer Nikotin- und Tabakprodukte ermöglicht. Ihre daraus resultierenden markierten thermischen Zersetzungsprodukte wurden im Rauch und dementsprechend bei den Rauchern gefunden, die als Positivkontrolle dienten, während die Anzahl und die Menge der thermischen Zersetzungsprodukte im Aerosol bzw. im Vaper deutlich geringer sind. Die Verknüpfung der Daten für die nativen (unmarkierten) und die markierten Biomarker ergab, dass Vaping eine geringe

Expositionsquelle für diese Substanzen darstellt. Die thermische Zersetzung von Propylenglykol und Glycerin zu anderen Gefahrenstoffen wie z.B. Crotonaldehyd findet nur während der Pyrolyse in Zigaretten statt und nicht durch Vapen.

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Validation data

Table 17: Validation data of PG/G/Nic in aerosol/smoke and e-liquid

Parameter		Glycerol				Propylene glycol				Nicotine			
LOD		3.4 µg/ml				0.4 µg/ml				0.2 µg/ml			
LLOQ		30 µg/ml				30 µg/ml				0.9 µg/ml			
Calibration range (CV [%] of 3 calibrations)		30 – 1000 µg/ml (5.6)				30 – 1000 µg/ml (5.1)				0.9 – 30 µg/ml (3.0)			
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 97 - 115%				No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 109 - 113%				No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 97 - 108%			
Accuracy (A) and Precision (P) after dilution (N=3)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	99	109	101		107	108	103		101	109	120	
	P [%]	7.1	9.6	2.4		6.6	3.8	3.3		9.1	9.8	0.0	
Matrix 2	**A [%]**	103	101	102		104	111	110		103	103	108	
	P [%]	5.7	3.3	1.2		2.1	4.6	1.5		2.6	4.2	6.7	
Matrix 3	**A [%]**	101	108	100		106	114	103		103	113	113	
	P [%]	8.0	7.0	0.2		2.6	4.1	4.2		2.8	3.8	2.8	
Carry over		1000 µg/ml: < LOD				1000 µg/ml: < LOD				1000 µg/ml: < LOD			
Accuracy (A) and Precision (P)		**LLOQ**	**Low**	**Med**	**High**	**LLOQ**	**Low**	**Med**	**High**	**LLOQ**	**Low**	**Med**	**High**
Intra-day 1 (N = 5)	**A [%]**	111	111	112	108	111	111	114	101	104	104	106	96
	P [%]	2.6	2.5	1.5	1.5	1.3	1.5	1.0	1.2	2.2	4.0	2.7	1.2
Intra-day 2 (N = 5)	**A [%]**	108	107	113	109	83	98	113	107	109	112	105	95
	P [%]	6.6	4.5	1.7	5.3	6.0	8.1	1.4	5.6	3.0	3.3	5.8	4.7
Intra-day 3 (N = 5)	**A [%]**	107	110	113	103	87	95	109	100	118	105	106	102
	P [%]	6.0	5.0	1.6	7.1	4.4	5.1	2.3	3.6	1.5	3.3	2.4	1.3
Inter-day (N = 15)	**A [%]**	109	109	113	107	94	101	112	103	110	107	106	98
	P [%]	5.3	4.2	1.6	5.3	14.2	8.8	2.6	4.9	5.8	4.6	3.8	4.2
Recovery [%] (N=6)		-	95	101	101	-	104	106	99	-	93	99	99
Stability (N=3)	**Short term [%]** (24h, 21°C)	-	106	-	110	-	101	-	100	-	102	-	94
	Freeze-thaw [%] (1 cycle)	-	113	-	105	-	103	-	101	-	98	-	97
	Postpreparative stability [%] (16 days, autosampler)	-	109	-	109	-	100	-	101	-	107	-	96
Re-Injection (CV[%]) (N=3 at 3 different days)		-	8.0	-	9.0	-	8.4	-	2.6	-	3.7	-	1.8
Matrix effect [%]		-	108	-	98	-	96	-	98	-	95	-	91

Table 18: Validation data of formaldehyde, acetaldehyde and acrolein in aerosol/smoke and e-liquid

Parameter		Formaldehyde			Acetaldehyde			Acrolein			
LOD		0.03 ng/ml			0.03 ng/ml			0.03 ng/ml			
LLOQ		0.1 ng/ml			0.1 ng/ml			0.1 ng/ml			
Calibration range (CV [%] of 3 calibrations)		0.1 – 500 ng/ml (2.0)			0.1 – 500 ng/ml (7.0)			0.1 – 500 ng/ml (2.2)			
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 106 - 109% Quan/Qual Ratio: 80 – 98 %			No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 98 – 115 % Quan/Qual Ratio: 97 - 119 %			No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 96 - 103% Quan/Qual Ratio: 97 - 120 %			
Accuracy (A) and Precision (P) after dilution (N=3)		Dilution 1	Dilution 2	Dilution 3	Dilution 1	Dilution 2	Dilution 3	Dilution 1	Dilution 2	Dilution 3	
Cigarette (1/10; 1/100; 1/1000)	**A [%]**	111	86	-****	99	95	-****	100	95	96	
	P [%]	0.5	3.9	-****	0.5	2.0	-****	0.4	0.7	7.3	
E-Cigarette (1/5; 1/50; 1/500)	**A [%]**	101	110	109	98	98	96	96	96	94	
	P [%]	1.1	3.3	4.1	1.2	2.8	0.4	1.1	2.9	3.2	
Carry over		500 ng/ml: < LOD			500 ng/ml: < LOD			500 ng/ml: < LOD			
		LLOQ (0.1 ng/ml)	**Low** (2 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)	**LLOQ** (0.1 ng/ml)	**Low** (2 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)	**LLOQ** (0.1 ng/ml)	**Low** (2 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)
Stability (N=3) **Short term [%]** (24h, 21°C)		-	94	-	102	-	97	-	100	-	102	-	102
Freeze-thaw [%] (3 Cycles)		-	108 (1:10) 113 (und.)	-	102 (1:10) 100 (und.)	-	107 (1:10) 114 (und.)	-	97 (1:10) 97 (und.)	-	108 (1:10) 105 (und.)	-	97 (1:10) 95 (und.)
Post preparative stability [%] (10 days, autosampler)		No post preparative stability There is an enrichment as soon as the vials are broached. If the sample must be measured repeatedly, a new dilution must be made from the original sample. In addition, the blank must also be determined again.				-	100	-	108	-	96	-	109
Stability of stock solution [%] (2,5 Years)		102 %				104 %				102 %			
Re-Injection (CV [%]) (N=3 at 3 different days)		-	89***	-	2.4	-	2.1	-	4.7	-	4.3	-	1.9
Matrix effekt [%] (E-Cigarette)		-	112**	-	99	-	101**	-	95	-	111**	-	99
Matrix effekt [%] (Cigarette)		-	109**	-	101	-	112**	-	99		109**		102
Matrix effekt [%] (DNPH-Solution)		-	102**	-	97	-	95**	-	94		103**	-	97
Re-Injection (CV [%]) (N=3 at 3 different days)		-	105**	-	100	-	103**	-	96		108**		100

Table 18 Continuation: Validation data of formaldehyde, acetaldehyde and acrolein in aerosol/smoke and e-liquid

Accuracy (A) and Precision (P)		LLOQ (0.1 ng/ml)	Low (0.25 ng/ml)	Med (5 ng/ml)	High (200 ng/ml)	LLOQ (0.1 ng/ml)	Low (0.25 ng/ml)	Med (5 ng/ml)	High (200 ng/ml)	LLOQ (0.1 ng/ml)	Low (0.25 ng/ml)	Med (5 ng/ml)	High (200 ng/ml)
Intra-day 1 (N = 5), 1:100	**A [%]**	-*	93	106	109	88	95	104	111	91	102	103	101
	P [%]	-*	14.3	3.0	2.1	3.0	2.4	1.1	2.0	8.1	3.7	2.6	3.1
Intra-day 2 (N = 5), 1:100	**A [%]**	-*	102	95	111	95	101	105	109	98	99	102	107
	P [%]	-*	9.8	6.2	1.3	8.4	2.1	1.2	0.8	8.0	3.7	2.5	1.7
Intra-day 3 (N = 5), 1:100	**A [%]**	-*	98	97	102	96	105	106	107	90	91	95	92
	P [%]	-*	6.6	8.1	7.0	4.1	3.4	3.8	7.1	10.3	6.5	4.1	6.8
Inter-day (N = 15), 1:100	**A [%]**	-*	97	99	108	93	100	105	109	93	98	100	100
	P [%]	-*	10.5	7.4	5.3	6.7	5.2	2.3	4.2	9.1	6.6	4.6	7.6
Intra-day 1 (N = 5), 1:10	**A [%]**	-*	97	106	107	103	99	105	107	105	105	99	102
	P [%]	-*	13.1	2.2	2.8	15.1	6.3	2.4	3.7	10.2	3.6	2.5	3.9
Intra-day 2 (N = 5), 1:10	**A [%]**	-*	105	98	108	92	105	104	105	110	109	107	113
	P [%]	-*	12.6	4.1	1.8	12.1	12.0	2.0	1.7	4.4	3.8	1.2	1.2
Intra-day 3 (N = 5), 1:10	**A [%]**	-*	100	100	109	109	104	112	114	93	96	95	93
	P [%]	-*	12.6	5.0	2.7	13.6	10.5	2.6	1.5	10.0	5.2	2.1	1.2
Inter-day (N = 15), 1:10	**A [%]**	-*	100	100	109	101	103	107	109	102	103	100	103
	P [%]	-*	12.6	5.0	2.7	14.6	9.7	4.1	4.3	10.7	6.5	5.8	8.4
Intra-day 1 (N = 5) undiluted	**A [%]**	-*	-*	105	108	-*	-*	100	107	113	109	110	103
	P [%]	-*	-*	2.4	3.5	-*	-*	3.6	2.8	6.7	4.1	1.5	2.7
Intra-day 2 (N = 5) undiluted	**A [%]**	-*	-*	94	112	-*	-*	103	113	104	87	115	98
	P [%]	-*	-*	8.8	1.1	-*	-*	3.0	3.6	10.8	7.3	0.4	4.2
Intra-day 3 (N = 5) undiluted	**A [%]**	-*	-*	95	109	-*	-*	108	103	100	100	96	93
	P [%]	-*	-*	7.6	3.5	-*	-*	5.9	4.4	7.6	8.3	3.0	4.0
Inter-day (N = 5) undiluted	**A [%]**	-*	-*	98	110	-*	-*	104	107	106	98	107	98
	P [%]	-*	-*	8.1	3.2	-*	-*	5.3	5.0	9.5	11.4	7.6	5.3

* Due to the high background level in the DNPH solution, these levels could not be determined. However, it was shown that the concentration in ACN is reproducible.
** Due to the high background exposure to the analyte, the matrix effect was only determined using the internal standard.
*** The sample cannot be reinjected in the low range. It must be diluted again from the original solution. In addition, a blank of the measuring day must also be diluted with new.
**** Dilution factor too high

Table 19: Validation data of crotonaldehyde and methacrolein in aerosol/smoke and e-liquid

Parameter		Crotonaldehyde				Methacrolein			
LOD		0.03 ng/ml				0.02 ng/ml			
LLOQ		0.1 ng/ml				0.1 ng/ml			
Calibration range (CV [%] of 3 calibrations)		0.1 – 500 ng/ml (3.9)				0.1 – 500 ng/ml (5.5)			
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 107 - 110% Quan/Qual Ratio: 95 - 105%				No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 93 – 98 % Quan/Qual Ratio: 83 – 117 %			
Accuracy (A) and Precision (P) after dilution (N=3)		Dilution 1	Dilution 2	Dilution 3		Dilution 1	Dilution 2	Dilution 3	
Cigarette (1/10; 1/100; 1/1000)	**A [%]**	99	94	-*		105	95	-*	
	P [%]	2.8	1.1	-*		1.5	10.2	-*	
E-Cigarette (1/5; 1/50; 1/500)	**A [%]**	103	104	99		95	93	92	
	P [%]	3.1	1.3	2.1		2.0	3.0	3.5	
Carry over		500 ng/ml: < LOD				500 ng/ml: < LOD			
Accuracy (A) and Precision (P)		**LLOQ** (0.1 ng/ml)	**Low** (0.25 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)	**LLOQ** (0.1 ng/ml)	**Low** (0.25 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)
Intra-day 1 (N = 5), 1:100	**A [%]**	94	100	104	110	95	93	90	105
	P [%]	4.6	7.0	4.1	3.7	9.9	5.4	4.1	3.2
Intra-day 2 (N = 5), 1:100	**A [%]**	97	102	102	101	95	100	98	101
	P [%]	7.2	1.6	1.0	1.0	6.6	4.0	1.7	1.1
Intra-day 3 (N = 5), 1:100	**A [%]**	98	98	110	105	100	100	107	105
	P [%]	4.2	5.5	3.9	7.0	0.8	2.5	3.9	7.1
Iter-day (N**n** = 15), 1:100	**A [%]**	96	100	105	105	97	98	99	104
	P [%]	5.5	5.0	4.5	5.6	6.8	5.1	8.0	4.7
Intra-day 1 (N = 5), 1:10	**A [%]**	107	96	106	107	97	91	94	105
	P [%]	4.2	7.2	3.0	2.8	5.0	5.8	2.2	5.0
Intra-day 2 (N = 5), 1:10	**A [%]**	102	104	102	101	100	100	98	102
	P [%]	1.2	4.3	0.7	1.6	1.8	4.3	0.5	1.5
Intra-day 3 (N = 5), 1:10	**A [%]**	100	102	107	104	100	104	106	105
	P [%]	8.0	5.0	2.1	1.4	5.3	4.2	2.1	1.7
Inter-day (N = 15), 1:10	**A [%]**	103	100	105	104	100	99	100	104
	P [%]	5.8	6.2	3.1	3.2	4.3	7.1	5.3	3.2
Intra-day 1 (N = 5) undiluted	**A [%]**	94	99	106	113	119	109	100	108
	P [%]	6.1	1.0	1.7	2.7	10.2	4.7	2.3	2.5
Intra-day 2 (N = 5) undiluted	**A [%]**	104	111	99	105	91	99	99	108
	P [%]	8.2	7.4	2.9	4.1	5.7	7.8	0.8	3.6
Intra-day 3 (N = 5) undiluted	**A [%]**	88	102	105	102	114	109	107	106
	P [%]	2.2	1.0	2.6	4.3	4.9	2.5	2.7	3.8
Inter-day (N = 5) undiluted	**A [%]**	95	104	103	107	108	106	102	107
	P [%]	9.3	6.8	4.0	5.7	13.9	6.5	3.9	3.2
Stability (N=3)	**Short term [%]** (24h, 21°C)	-	110	-	104	-	111	-	100
	Freeze-thaw [%] (3 Cycles)	-	108 (1:10) 105 (und.)	-	96 (1:10) 95 (und.)	-	115 (1:10) 115 (und.)	-	100 (1:10) 100 (und.)
	Postpreparative stability [%] (10 days, autosampler)	-	89	-	98	-	87	-	98
	Stability of stock solution [%]	110 %				-			
Re-Injection (CV [%]) (N=3 at 3 different days)		-	8.4	-	7.4	-	6.1	-	5.0
Matrix effekt [%] (E-Cigarette)		-	115**	-	95	-	115**	-	97
Matrix effekt [%] (Cigarette)		-	119**	-	101	-	119**	-	102
Matrix effekt [%] (DNPH-Solution)		-	115	-	98	-	116	-	99

* Dilution factor too high
** Due to the high background concentration of the analytes the internal standards were used to determine the matrix effect.

Table 20: Validation data of acetone and propionaldehyde in aerosol/smoke and e-liquid

Parameter		**Aceton**				**Propionaldehyde**			
LOD		0.03 ng/ml				0.01 ng/ml			
LLOQ		0.1 ng/ml				0.1 ng/ml			
Calibration range (CV [%] of 3 calibrations)		0.1 – 500 ng/ml (5.9)				0.1 – 500 ng/ml (2.6)			
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 100 - 104% Quan/Qual Ratio: 91 - 115%				No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 102 - 107 % Quan/Qual Ratio: 90 - 103 %			
Accuracy (A) and Precision (P) after dilution (N=3)		Dilution 1	Dilution 2	Dilution 3		Dilution 1	Dilution 2	Dilution 3	
Cigarette (1/10; 1/100; 1/1000)	**A [%]**	103	99	-*		97	94	95	
	P [%]	1.4	1.6	-*		0.9	3.4	0.2	
E-Cigarette (1/5; 1/50; 1/500)	**A [%]**	87	88	93		95	96	99	
	P [%]	3.1	1.3	2.1		2.0	3.0	3.5	
Carry over		500 ng/ml: < LOD				500 ng/ml: < LOD			
Accuracy (A) and Precision (P)		**LLOQ** (0.1 ng/ml)	**Low** (0.25 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)	**LLOQ** (0.1 ng/ml)	**Low** (0.25 ng/ml)	**Med** (5 ng/ml)	**High** (200 ng/ml)
Intra-day 1 (N = 5), 1:100	**A [%]**	88	108	100	93	93	103	102	105
	P [%]	8.6	3.0	2.3	2.7	11.6	5.2	1.8	2.1
Intra-day 2 (N = 5), 1:100	**A [%]**	103	113	111	106	103	114	112	111
	P [%]	14.6	7.6	2.3	2.1	10.7	2.1	2.0	1.0
Intra-day 3 (N = 5), 1:100	**A [%]**	102	98	98	99	104	104	109	109
	P [%]	14.3	12.8	4.5	7.1	4.7	4.5	4.3	6.7
Iter-day (N = 15), 1:100	**A [%]**	98	106	103	99	100	107	108	108
	P [%]	14.4	10.0	6.2	7.2	10.0	6.2	5.0	4.5
Intra-day 1 (N = 5), 1:10	**A [%]**	103	91	101	92	80	85	100	101
	P [%]	12.3	8.9	4.5	1.6	7.2	5.7	1.1	3.1
Intra-day 2 (N = 5), 1:10	**A [%]**	99	102	110	109	95	111	106	110
	P [%]	15.1	13.7	2.7	1.6	13.4	10.0	1.0	1.6
Intra-day 3 (N = 5), 1:10	**A [%]**	102	104	97	100	103	101	105	108
	P [%]	14.7	5.9	2.3	2.2	9.1	6.8	1.4	1.7
Inter-day (N = 15), 1:10	**A [%]**	101	99	103	100	93	99	103	106
	P [%]	13.1	10.9	6.4	7.4	14.4	13.5	2.9	4.3
Intra-day 1 (N = 5) undiluted	**A [%]**	-**	-**	90	108	-**	-**	97	100
	P [%]	-**	-**	7.1	2.9	-**	-**	3.0	1.8
Intra-day 2 (N = 5) undiluted	**A [%]**	-**	-**	100	94	-**	-**	108	113
	P [%]	-**	-**	12.5	1.9	-**	-**	2.4	1.2
Intra-day 3 (N = 5) undiluted	**A [%]**	-**	-**	109	97	-**	-**	108	107
	P [%]	-**	-**	14.3	2.3	-**	-**	3.7	3.7
Inter-day (N = 5) undiluted	**A [%]**	-**	-**	100	100	-**	-**	104	107
	P [%]	-**	-**	14.0	6.4	-**	-**	5.8	5.6
Stability (N=3)	**Short term [%]** (24h, 21°C)	-	95	-	105	-	111	-	100
	Freeze-thaw [%] (3 Cycles)	-	105 (1:10) 111+ (unv.)	-	108 (1:10) 115+(unv.)	-	107 (1:10) 96 (unv.)	-	103 (1:10) 101 (unv.)
	Postpreparative stability [%] (10 days, autosampler)	-	92	-	101	-	104	-	109
	Stability of stock solution [%]	-				-			
Re-Injection (CV [%]) (N=3 at 3 different days)		-	2.4	-	4.0	-	4.0	-	2.0
Matrix effekt [%] (E-Cigarette)		-	2.4	-	4.0	-	4.0	-	2.0
Matrix effekt [%] (Cigarette)		-	93***	-	95	-	99***	-	95
Matrix effekt [%] (DNPH-Solution)		-	100***	-	105	-	110***	-	102

* Dilution factor too high

** Due to the high background contamination in the DNPH solution, the levels were not applicable but it was shown that the concentrations were reproduciable in ACN. (Data not shown)

*** Due to the high background concentration of the analytes the internal standards were used to determine the matrix effect.

+ The stability was only proven for one cycle. After the first cycle an enrichment was observed. The enrichment was not observed in the dilution 1:10. Therefore a sufficient amount of an aliquot of the dilution 1:10 needs to be prepared before the first freeze-thaw cycle.

Table 21: Validation data of N-Nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in aerosol/smoke and e-liquid

Parameters		**NNN**				**NNK**			
LOD		0.03 ng per filter				0.02 ng per filter			
LLOQ		0.1 ng per filter				0.1 ng per filter			
calibration range (CV of 3 calibrations [%])		0.1 - 500 ng per filter (2.8)				0.1 - 500 ng per filter (8.5)			
Selectivity		No interference at the retention time of the analyt and the internal standard Accuracy of spiking: 89 - 93% Quan/Qualifier Ratio: 94 - 100%				No interference at the retention time of the analyt The interference at the retention time of the internal standard is < 0.1 % Accuracy of spiking: 88 - 98% Quan/Qualifier Ratio: 91 - 116%			
Verschleppung		200 ng per filter: < LOD				500 ng per filter: < LOD			
Accuracy & Presicion		**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)	**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)
Intra-day 1 (N = 5)	**A [%]**	113	101	94	107	111	95	95	105
	P [%]	6.2	9.0	5.7	3.2	7.0	5.8	4.2	2.2
Intra-day 2 (N = 5)	**A [%]**	108	91	89	103	99	98	93	102
	P [%]	6.6	5.4	2.9	1.2	5.7	6.0	4.5	1.8
Intra-day 3 (N = 5)	**A [%]**	97	93	91	102	117	108	91	101
	P [%]	8.4	6.5	1.7	1.6	1.9	6.0	2.8	1.8
Inter-day (N = 15)	**A [%]**	106	95	91	104	109	101	93	102
	P [%]	9.1	8.2	4.2	3.0	8.5	7.8	3.9	2.4
Recovery [%] (N=6)		-	96	94	98	-	111	103	109
Stability (N=3)		dry filter pads		extracted filter pads		dry filter pads		extracted filter pads	
		0.2 ng	50 ng	0.2 ng	50 ng	0.2 ng	50 ng	0.2 ng	50 ng
	Short term [%] (dry filter pads24h, 21°C)	88	90	103	93	97	94	106	97
	Freeze-thaw cycle [%] (6 cycles)	93	109	86	102	93	90	104	109
	Post-preparation stability [%] (14 Tage, Autosampler)	-	-	105	94	-	-	95	97
	Stability of the stock solution [%]	1.32 mg/ml: 87%				1 mg/ml: 111%			
Re-Injection (CV [%]) (N=3 at 3 different days)		0.2 ng: 9.6		200 ng: 2.3		0.2 ng: 9.7		200 ng: 1.0	
Matrix effect [%] (Filter)		8 ng: 99		80 ng: 101		10 ng: 98%		100 ng: 97%	
Matrix effect [%] (Aerosol)		8 ng: 101		80 ng: 92		10 ng: 102%		100 ng: 99%	
Matrix effect [%] (Smoke)		8 ng: 23		80 ng: 22		10 ng:15%		100 ng: 21%	

Table 22: Validation data of N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB) in aerosol/ smoke and e-liquid

Parameters		**NAB**				**NAT**			
LOD		0.02 ng per filter				0.03 ng per filter			
LLOQ		0.1 ng per filter				0.1 ng per filter			
calibration range (CV of 3 calibrations [%])		0.1 - 200 ng per filter (7.4)				0.1 - 500 ng per filter (0.8)			
Selectivity		No interference at the retention time of the analyt and the internal standard Accuracy of spiking: 87 - 92% Quan/Qualifier Ratio: 95-111%				No interference at the retention time of the analyt and the internal standard Accuracy of spiking: 100 - 104% Quan/Qualifier Ratio: 103 - 112%			
Verschleppung		200 ng per filter: < LOD				500 ng per filter: < LOD			
Accuracy & Presicion		**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)	**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)
Intra-day 1 (N = 5)	**A [%]**	114	101	96	108	107	106	97	106
	P [%]	3.5	4.3	3.6	1.5	11.5	5.0	2.1	2.5
Intra-day 2 (N = 5)	**A [%]**	109	96	92	104	96	96	99	109
	P [%]	5.4	6.7	2.7	3.2	13.5	9.1	1.7	1.5
Intra-day 3 (N = 5)	**A [%]**	105	96	90	100	106	99	101	110
	P [%]	11.8	5.3	1.7	2.0	11.0	8.6	1.2	1.7
Inter-day (N = 15)	**A [%]**	109	97	92	104	103	100	99	108
	P [%]	7.9	5.7	3.9	3.9	12.1	8.3	2.5	2.5
Recovery [%] (N=6)		-	101	94	96	-	98	94	97
Stability (N=3)		dry filter pads		extracted filter pads		dry filter pads		extracted filter pads	
		0.2 ng	50 ng	0.2 ng	50 ng	0.2 ng	50 ng	0.2 ng	50 ng
	Short term [%] (dry filter pads24h, 21°C)	76	78	94	94	85	86	93	104
	Freeze-thaw cycle [%] (6 cycles)	105	93	93	109	107	112	95	102
	Post-preparation stability [%] (14 Tage, Autosampler)	-	-	92	96	-	-	95	97
	Stability of the stock solution [%]	1.04 mg/ml: 109.6% (6.5 Jahre)				1.04 mg/ml: 109.6% (6.5 Jahre)			
Re-Injection (CV [%]) (N=3 at 3 different days)		0.2 ng: 7.4		200 ng: 1.6		0.2 ng: 11.3		200 ng: 1.7	
Matrix effect [%] (Filter)		8 ng: 102		80 ng: 101		7.5 ng: 99%		75 ng: 101%	
Matrix effect [%] (Aerosol)		8 ng: 99		80 ng: 99		7.5 ng: 90%		75 ng: 86%	
Matrix effect [%] (Smoke)		8 ng: 23		80 ng: 19		7.5 ng:33%		75 ng: 27%	

Table 23: Validation data of glycerol and propylene glycol in plasma and urine

		Glycerol					
Parameter		**Plasma**			**Urine**		
LOD		0.01 µg/ml					
LLOQ (CV at LLOQ (N = 6))		0.1 µg/ml (5.0 %)					
Calibration range		0.1 – 150 µg/ml					
Selectivity		No interference at the retention time of the analyte and the internal standard Quan/Qual Ratio: 98 - 108%			No interference at the retention time of the analyte and the internal standard Quan/Qual Ratio: 83 – 117 %		
Carry over		150 µg/ml: < LOD					
Accuracy in water (N=5) [%]		**Low** (0.5 µg/ml)		**Medium** (5 µg/ml)		**High** (100 µg/ml)	
		96		92		106	
Precision (N=6) [%]		**Low** (0.3 µg/ml)	**Medium** (3.2 µg/ml)	**High** (72 µg/ml)	**Low** (0.3 µg/ml)	**Medium** (3.2 µg/ml)	**High** (72 µg/ml)
	Intra-day	9.6	4.0	4.8	10.5	11.3	11.8
	Inter-day	15.2	14.5	3.0	5.6	10.2	9.3
Stability (N=3)	**Short term [%]** (16h, 21°C)	94	-	102	96	-	100
	Freeze-thaw [%] (6 Cycles)	89	-	94	82	-	91
	Postpreparative stability [%] (9 days, autosampler, 10°C)	91	-	110	112	-	100
	Stability of stock solution	24 months			24 months		
	Longterm stability of matrix samples	16 months			16 months		
Re-Injection (CV [%]) (N=3 at 3 different days)		7.6	-	3.4	4.0	-	6.6

		Propylene glycol					
Parameter		**Plasma**			**Urine**		
LOD		0.02 µg/ml					
LLOQ (accuracy at LLOQ (N = 6 * 3))		0.1 µg/ml (92.2 %)			0.1 µg/ml (100.1 %)		
Calibration range		0.1 – 150 µg/ml					
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 89 – 108 % Quan/Qual Ratio: 86 - 109%					
Carry over		150 µg/ml: < LOD					
Accuracy in water (N=5) [%]		**Low** (0.5 µg/ml)		**Medium** (5 µg/ml)		**High** (100 µg/ml)	
		97		101		100	
Precision (N=6) [%]		**Low** (0.9 µg/ml)	**Medium** (5.2 µg/ml)	**High** (84 µg/ml)	**Low** (0.3 µg/ml)	**Medium** (7.8 µg/ml)	**High** (117.2 µg/ml)
	Intra-day	8.5	3.8	2.0	18.0	9.4	7.8
	Inter-day	11.0	6.0	7.1	13.1	3.1	8.8
Stability (N=3)	**Short term [%]** (16h, 21°C)	104	-	109	109	-	90
	Freeze-thaw [%] (6 Cycles)	101	-	107	90	-	86
	Postpreparative stability [%] (9 days, autosampler, 10°C)	95	-	96	84	-	97
	Stability of stock solution [%]	24 months			24 months		
	Longterm stabilty of matrix samples	21 months			8 months		
Re-Injection (CV [%]) (N=3 at 3 different days)		4.5	-	2.0	8.0	-	4.6

Table 25: Validation data of thiazolidine carboxylic acid in plasma and urine

Parameters		**Methods characteristics for the determination of TCA in plasma and urine**							
		Plasma				**Urine**			
LOD		0.07 ng/ml (QTRAP® 6500+)				0.13 ng/ml (QTRAP® 6500+)			
LLOQ		0.5 ng/ml				0.5 ng/ml			
calibration range		0.5 - 200 ng/ml				0.5 - 200 ng/ml			
Selectivity		No interference at the retention time of the internal standard Accuracy of spiking: 87 – 112 Quan/Qualifier Ratio: 96 - 99%				No interference at the retention time the internal standard Accuracy of spiking: 88 - 97% Quan/Qualifier Ratio: 87-93%			
Carry over		200 ng/ml: < LOD							
Accuracy (A) & Presicion (P)		**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)	**LLOQ** (0.1 ng)	**Low** (0.2 ng)	**Med** (5 ng)	**High** (200 ng)
Intra-day 1 (N = 5)	**A [%]**	93	92	87	88	94	99	95	97
	P [%]	9.0	2.4	4.6	1.5	10.4	5.4	3.8	1.4
Intra-day 2 (N = 5)	**A [%]**	100	92	102	95	92	103	102	101
	P [%]	9.7	3.5	5.2	2.6	11.0	4.5	4.2	1.5
Intra-day 3 (N = 5)	**A [%]**	102	96	98	85	104	104	99	107
	P [%]	4.9	2.4	1.3	2.0	4.5	4.1	1.2	2.8
Inter-day (N = 15)	**A [%]**	99	93	96	89	97	102	98	101
	P [%]	8.6	3.3	7.7	5.3	9.9	4.8	4.5	4.8
Recovery [%] (N=6)		Not applicable				-	94	83	84
Stability (N=3)		Med (5 ng/ml)		High (50 ng/ml)		Med		High	
	Short term [%] (dry filter pads24h, 21°C)	113		101		99		101	
	Freeze-thaw cycle [%] (6 cycles)	101		97		99		102	
	Post-preparation stability [%] (5 days, Autosampler)	107		96		98		95	
	Stability of the stock solution [%]	10.42 mg/ml: 99% (21 months)							
Re-Injection (CV [%]) (N=3 at 3 different days)		2 ng: 4.4		50 ng: 1.3		2 ng: 4.4		50 ng: 1.3	
Matrix effect [%]		- 30 %				- 50 %			
Accuracy (A) and Precision (P) after dilution (N = 3)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	101	98	89		94	87	89	
	P [%]	5.8	4.6	11		14.2	14.1	12.6	
Matrix 2	**A [%]**	100	104	103		96	110	105	
	P [%]	1.6	7.0	11.1		8.2	13.8	11.9	
Matrix 3	**A [%]**	103	102	94		95	105	90	
	P [%]	3.0	11.03	14.8		5.2	14.5	2.8	

Table 24: Validation data of nicotine, cotinine and OH-cotinine in plasma

Parameter		Nikotin			Cotinin			OH-Cotinin			
LOD		0.02 ng/ml			0.003 ng/ml			0.02 ng/ml			
LLOQ		0.1 ng/ml			0.5 ng/ml			0.5 ng/ml			
Calibration range (CV [%] of 3 calibrations)		0.1 - 100 ng/ml (0.3)			0.5 – 500 ng/ml (6.2)			0.5 – 500 ng/ml (0.5)			
Selectivity		No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 96 - 111% Quan/Qual Ratio: 94 – 100 %			No interference at the retention time of the analyte and the internal standard Accuracy of spiking at LLOQ: 110 - 120% Quan/Qual Ratio: 90 - 105%			No interference at the retention time of the analyte and the internal standard Accuracy of spiking: 80 - 94% Quan/Qual Ratio:100 - 113 %			
Accuracy (A) and Precision (P) after dilution (N=3)		Dilution 1 (1/2)	Dilution 2 (1/4)	Dilution 3 (1/10)	Dilution 1 (1/2)	Dilution 2 (1/4)	Dilution 3 (1/10)	Dilution 1 (1/2)	Dilution 2 (1/4)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	102	104	98	98	106	105	98	103	98	
	P [%]	5.6	6.7	2.0	1.4	6.7	5.0	4.5	9.1	1.2	
Matrix 2	**A [%]**	102	109	111	100	104	111	98	97	96	
	P [%]	5.0	2.7	1.8	4.7	6.5	2.8	4.6	5.2	5.9	
Matrix 3	**A [%]**	100	112	113	94	95	95	92	94	93	
	P [%]	6.4	3.4	3.6	8.9	2.4	6.0	5.7	2.4	4.4	
Carry over		100 ng/ml: < LOD			500 ng/ml: < LOD			500 ng/ml: < LOD			
Accuracy (A) and Precision (P)		**LLOQ** (0,1 ng/ml)	**Low** (0,3 ng/ml)	**Med** (5 ng/ml)	**High** (50 ng/ml)	**LLOQ** (0,5 ng/ml)	**Low** (1.5 ng/ml)	**Med** (25 ng/ml)	**High** (250 ng/ml)	**LLOQ** (0,5 ng/ml)	**Low** (1.5 ng/ml)	**Med** (25 ng/ml)	**High** (250 ng/ml)
Intra-day 1 (N = 5)	**A [%]**	113	110	114	112	91	87	103	109	90	94	110	111
	P [%]	9.7	7.5	1.1	2.0	4.5	2.7	3.7	3.7	6.8	1.8	1.5	0.9
Intra-day 2 (N = 5)	**A [%]**	94	113	105	106	99	83	95	100	86	86	105	107
	P [%]	16.0	3.1	6.1	3.4	4.9	4.0	2.9	2.1	9.2	1.3	3.4	3.2
Intra-day 3 (N = 5)	**A [%]**	93	95	106	107	102	92	97	99	100	94	100	97
	P [%]	8.3	5.8	3.1	2.2	8.4	4.9	0.8	1.9	10	3.8	2.8	2.7
Inter-day (N = 15)	**A [%]**	99.9	106	108	108	97	87	98	103	92	91	105	105
	P [%]	14.6	9.3	5.4	3.6	7.6	6.1	4.5	5.4	10.4	5.1	4.8	6.3
Recovery [%] (N=6)		-	59	65	65	-	56	65	67	-	29	32	39
Stabilität (N=3)	**Short term [%]** (24h, 21°C)	-	103	-	93	-	114	-	104	-	103	-	99
	Freeze-thaw [%] (6 Cycles)	-	109	-	107	-	101	-	99	-	111	-	115
	Long term stbility in matrix [%] (14 months)	-	110	-	113	-	115	-	107	-	-	-	-
	Stanility of stock solution [%] (17 months)	-	-	114*	-	-	-	120**	-	-	-	113*	-
	Post preparative stability (autosampler, 5 days)	-	109	-	107	-	113	-	112	-	97	-	112
Re-Injection (CV) [%] (N=3 at 3 different days)		-	4.4	-	3.2	-	8.4	-	2.6	-	4.4	-	3.2
Matrix effekt [%]	**Analyte**	-	94	-	104	-	88	-	89	-	99	-	102
	IS	-	106	-	91	-	96	-	97	-	111	-	97

* Nicotine and OH-cotinine in ACN should not be used for longer than 17 months.
** Cotinine in ACN is not stable for 17 months. A review within a shorter timeframe is necessary.

Table 26: Validation data of methyl thiazolidine carboxylic acid in plasma and urine

Parameters		**Methods characteristics for the determination of MTCA in plasma and urine**							
		Plasma				**Urine**			
LOD		0.1 ng/ml (QTRAP® 6500$^+$)				0.12 ng/ml (QTRAP® 6500$^+$)			
LLOQ		0.5 ng/ml				0.5 ng/ml			
calibration range		0.5 - 200 ng/ml				0.5 - 200 ng/ml			
Selectivity		No interference at the retention time of the internal standard Accuracy of spiking: 98 – 115 % Quan/Qualifier Ratio: 98 – 102 %				No interference at the retention time the internal standard Accuracy of spiking: 101 – 107 % Quan/Qualifier Ratio: 96 – 112 %			
Carry over		200 ng/ml: < LOD							
Accuracy (A) & Presicion (P)		**LLOQ** (0.5 ng)	**Low** (1.5 ng)	**Med** (10 ng)	**High** (100 ng)	**LLOQ** (0.5 ng)	**Low** (1.5 ng)	**Med** (10 ng)	**High** (100 ng)
Intra-day 1 (N = 5)	**A [%]**	99	96	100	95	90	92	97	115
	P [%]	2.4	5.1	5.0	3.4	2.1	1.4	2.8	0.5
Intra-day 2 (N = 5)	**A [%]**	94	94	94	99	94	99	106	110
	P [%]	7.9	5.3	2.5	5.3	4.5	6.2	2.1	1.9
Intra-day 3 (N = 5)	**A [%]**	103	101	112	95	92	99	110	111
	P [%]	8.3	8.1	4.5	2.4	3.4	6.6	2.1	0.8
Inter-day (N = 15)	**A [%]**	99	97	102	96	92	97	104	112
	P [%]	7.4	6.8	8.8	4.2	3.8	6.2	6.0	2.2
Recovery [%] (N=6)		Not applicable				-	55	55	
Stability (N=3)		Med (5 ng/ml)		High (50 ng/ml)		Med		High	
	Short term [%] (dry filter pads24h, 21°C)	98		88		94		103	
	Freeze-thaw cycle [%] (6 cycles)	106		102		98		95	
	Post-preparation stability [%] (5 days, Autosampler)	114		91		97		102	
	Stability of the stock solution [%]	5 mg/ml: 85% (2 months); [21 months: 60 %]							
Re-Injection (CV [%]) (N=3 at 3 different days)		2 ng: 2.7		50 ng: 1.2		5		50 ng: 1.2	
Matrix effect [%]		- 33 %				- 33 %			
Accuracy (A) and Precision (P) after dilution (N = 3)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	86	93	104		96	114	101	
	P [%]	4.3	4.8	6.3		4.4	11.6	10.0	
Matrix 2	**A [%]**	96	100	106		105	108	101	
	P [%]	8.7	9.1	6.1		15.0	7.7	4.6	
Matrix 3	**A [%]**	101	90	101		101	100	91	
	P [%]	11.2	8.3	4.0		12.3	13.0	10	

Table 27: Validation data of thiazolidine carboxylic acid in plasma and urine

Parameters		**Methods characteristics for the determination of TCG in plasma and urine**							
		Plasma				**Urine**			
LOD		0.33 ng/ml (QTRAP® 6500$^+$)				0.25 ng/ml (QTRAP® 6500$^+$)			
LLOQ		1 ng/ml				1 ng/ml			
calibration range		1 - 400 ng/ml				1 - 400 ng/ml			
Selectivity		No interference at the retention time of the internal standard Accuracy of spiking: 93 – 115 % Quan/Qualifier Ratio:79 - 111 %				No interference at the retention time of the internal standard Accuracy of spiking: 88 - 114 % Quan/Qualifier Ratio: 86 – 114 %			
Carry over		400 ng/ml: < LOD							
Accuracy (A) & Presicion (P)		**LLOQ** (1 ng)	**Low** (3 ng)	**Med** (20 ng)	**High** (200 ng)	**LLOQ** (1 ng)	**Low** (3 ng)	**Med** (20 ng)	**High** (200 ng)
Intra-day 1 (N = 5)	**A [%]**	94	100	90	92	104	104	98	89
	P [%]	7.8	2.2	4.8	3.8	4.0	5.1	6.8	5.3
Intra-day 2 (N = 5)	**A [%]**	102	88	86	88	105	89	105	113
	P [%]	7.1	8.1	1.5	3.6	4.7	7.3	5.1	1.7
Intra-day 3 (N = 5)	**A [%]**	108	101	104	91	102	96	87	101
	P [%]	7.7	4.5	2.9	7.1	7.0	6.6	11.7	3.8
Inter-day (N = 15)	**A [%]**	101	96	93	90	104	96	97	101
	P [%]	9.0	8.0	8.9	5.0	5.1	8.7	10.7	10.8
Recovery [%] (N=6)		Not applicable				-	32	29	29
Stability (N=3)		Med (5 ng/ml)		High (50 ng/ml)		Med (5 ng/ml)		High (50 ng/ml)	
	Short term [%] (dry filter pads24h, 21°C)	89		94		106		103	
	Freeze-thaw cycle [%] (6 cycles)	95		87		90		86	
	Post-preparation stability [%] (5 days, Autosampler)	112		89		97		92	
Re-Injection (CV [%]) (N=3 at 3 different days)		2 ng: 5.2		50 ng: 7.3		2 ng: 5.2		50 ng: 7.3	
Matrix effect [%]		- 33 %				- 60%			
Accuracy (A) and Precision (P) after dilution (N = 3)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	101	92	94		100	87	84	
	P [%]	6.5	5.3	2.5		4.1	9.5	19.1	
Matrix 2	**A [%]**	99	94	95		99	101	93	
	P [%]	6.5	4.1	3.1		10.6	13.9	10.3	
Matrix 3	**A [%]**	104	94	95		108	94	86	
	P [%]	3.4	14.9	7.0		3.2	13.9	8.1	

Table 28: Validation data of methyl thiazolidine carboxylic acid in plasma and urine

Parameters		Methods characteristics for the determination of MTCG in plasma and urine							
		Plasma				Urine			
LOD		0.18 ng/ml (QTRAP® 6500+)				0.27 ng/ml (QTRAP® 6500+)			
LLOQ		1 ng/ml				1 ng/ml			
calibration range		1 - 400 ng/ml				1 - 400 ng/ml			
Selectivity		No interference at the retention time of the internal standard Accuracy of spiking: 88 – 107 % Quan/Qualifier Ratio: 83 – 112 %				No interference at the retention time of the internal standard Accuracy of spiking: 93 - 103 % Quan/Qualifier Ratio: 78 – 121 %			
Carry over		400 ng/ml: < LOD							
Accuracy (A) & Presicion (P)		**LLOQ** (1 ng)	**Low** (3 ng)	**Med** (20 ng)	**High** (200 ng)	**LLOQ** (1 ng)	**Low** (3 ng)	**Med** (20 ng)	**High** (200 ng)
Intra-day 1 (N = 5)	**A [%]**	99	114	104	101	83	85	94	103
	P [%]	9.9	4.2	6.5	5.6	3.7	12.5	4.4	5.5
Intra-day 2 (N = 5)	**A [%]**	94	87	93	101	90	88	99	92
	P [%]	6.1	5.9	11.0	4.9	9.8	3.2	11.9	7.3
Intra-day 3 (N = 5)	**A [%]**	85	88	104	111	86	93	96	92
	P [%]	2.8	2.9	3.5	2.7	6.9	5.4	3.9	3.7
Inter-day (N = 15)	**A [%]**	92	96	100	104	86	88	96	95
	P [%]	9.2	13.9	8.7	6.2	7.8	8.1	7.5	7.8
Recovery [%] (N=6)		Not applicable (n.a.)				-	25	23	29
Stability (N=3)		Med (3 ng/ml)		High (65 ng/ml)		Med (3 ng/ml)		High (85 ng/ml)	
	Short term [%] (dry filter pads24h, 21°C)	93		91		105		101	
	Freeze-thaw cycle [%] (6 cycles)	86		102		103		103	
	Post-preparation stability [%] (5 days, Autosampler)	93		98		94		97	
Re-Injection (CV [%]) (N=3 at 3 different days)		4 ng/ml: 13		35 ng/ml: 11		4 ng/ml: 13		35 ng/ml: 11	
Matrix effect [%]		- 33 %				-60 %			
Accuracy (A) and Precision (P) after dilution (N = 3)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)		Dilution 1 (1/2)	Dilution 2 (1/5)	Dilution 3 (1/10)	
Matrix 1	**A [%]**	112	103	89		n.a.	n.a.	n.a.	
	P [%]	5.8	4.7	5.0		n.a.	n.a.	n.a.	
Matrix 2	**A [%]**	110	103	92		n.a.	n.a.	n.a.	
	P [%]	9.8	9.2	4.7		n.a.	n.a.	n.a.	
Matrix 3	**A [%]**	113	88	88		n.a.	n.a.	n.a.	
	P [%]	9.4	3.3	9.3		n.a.	n.a.	n.a.	

Subject questionnaire for Part A

Protokoll
Part A

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

Sehr geehrter Proband,

bitte beantworten Sie uns die unten aufgeführten Fragen nach den Sessions 1, 5 und 10.

After Session 1

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen): ○ gut ○ zufriedenstellend ○ ungenügend
2. Die Inhalierbarkeit des erzeugten Dampfes war: ○ gut ○ zufriedenstellend ○ ungenügend
3. War die Temperatur der E-Zigarette während des Dampfens ○ angenehm ○ unangenehm, wenn ja warum?_____

Time: __:__	Initials:

After Session 5

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen): ○ gut ○ zufriedenstellend ○ ungenügend
2. Die Inhalierbarkeit des erzeugten Dampfes war: ○ gut ○ zufriedenstellend ○ ungenügend
3. War die Temperatur der E-Zigarette während des Dampfens ○ angenehm ○ unangenehm, wenn ja warum?_____

Time: __:__	Initials:

Protokoll
Part A

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

After Session 10

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen):

   ○ gut ○ zufriedenstellend ○ ungenügend

2. Die Inhalierbarkeit des erzeugten Dampfes war:

   ○ gut ○ zufriedenstellend ○ ungenügend

3. War die Temperatur der E-Zigarette während des Dampfens

   ○ angenehm ○ unangenehm, wenn ja warum?_____

Time: __:__	Initials:

Subject questionnaire for Part B

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

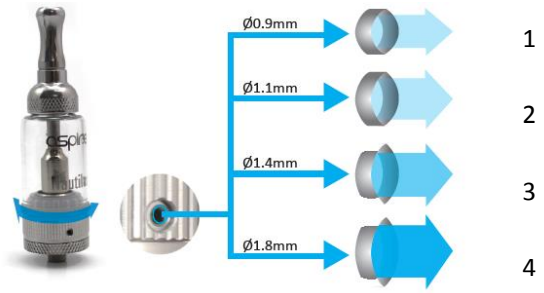
Datum:	Probandennummer:

Sehr geehrter Proband,

auf den folgenden Seiten finden Sie eine Art Tagebuch, in welchem Sie bitte die Zuganzahl und die Einstellungen an der E-Zigarette, sowie den Zeitpunkt der Urinabgabe eintragen. Außerdem bitten wir Sie, das Produkt anhand vier kurzer Fragen an jedem Studientag zu bewerten.

Hinweise:

a. Legen Sie die Kühlakkus am Vorabend bitte immer in den Tiefkühler.
b. Vermerken Sie bitte die Uhrzeit der morgendlichen Urinabgabe und stellen Sie das Urin-Gefäß aufrecht mit den Kühlakkus in die Kühltasche.
c. Vermerken Sie bitte in der unten stehenden Tabelle die Anzahl der Züge stundengenau (ein Strich pro Zug).
d. Halten Sie bitte außerdem den eingestellten Luftstrom (1-4) fest. Der Luftstrom lässt sich unterhalb des Tanks regulieren, wie die Abbildung zeigt.



e. Bringen Sie die Kühltasche mit dem Urin-Gefäß und das ausgefüllte Tagebuch zwischen 16 und 20 Uhr in die Klinik.
f. Bitte auf jedem Blatt des Tagebuchs unbedingt aktuelles Datum und Probanden-Nr. eintragen.
g. Bitte am Ende jedes Versuchstages die Fragen zur Bewertung der E-Zigarette beantworten

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

Tag -1

Uhrzeit	Anzahl der Züge	Leistung (in Watt)	Luftstrom (1-4)
16:00 – 17:00			
17:00 – 18:00			
18:00 – 19:00			
19:00 – 20:00			
20:00 – 21:00			
21:00 – 22:00			
22:00 – 23:00			
23:00 – 24:00			

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen):

   ○ gut ○ zufriedenstellend ○ ungenügend

2. Die Inhalierbarkeit des erzeugten Dampfes war:

   ○ gut ○ zufriedenstellend ○ ungenügend

3. Ich hatte während des abgelaufenen Tages das Bedürfnis wieder mein eigenes Produkt zu benutzen:

   ○ gar nicht ○ zeitweise ○ immer

4. Ich habe während des abgelaufenen Tages versucht den Geschmack und/oder die Inhalierbarkeit des Dampfes durch Veränderungen der Einstellungen an der E-Zigarette zu verbessern:

   ○ gar nicht ○ nur wenige Male ○ mehrfach

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

Tag 1

Zeitpunkt der Urinabgabe (morgens):_____

Zeitpunkt der Speichelabgabe (morgens):_____

Uhrzeit	Anzahl der Züge	Leistung (in Watt)	Luftstrom (1-4)
0:00 – 1:00			
1:00 – 2:00			
2:00 – 3:00			
3:00 – 4:00			
5:00 – 6:00			
6:00 – 7:00			
7:00 – 8:00			
8:00 – 9:00			
9:00 – 10:00			
10:00 – 11:00			
11:00 – 12:00			
12:00 – 13:00			
13:00 – 14:00			
14:00 – 15:00			
15:00 – 16:00			
16:00 – 17:00			
17:00 – 18:00			

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

18:00 – 19:00			
19:00 – 20:00			
20:00 – 21:00			
21:00 – 22:00			
22:00 – 23:00			
23:00 – 24:00			

5. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen):
   ○ gut ○ zufriedenstellend ○ ungenügend

6. Die Inhalierbarkeit des erzeugten Dampfes war:
   ○ gut ○ zufriedenstellend ○ ungenügend

7. Ich hatte während des abgelaufenen Tages das Bedürfnis wieder mein eigenes Produkt zu benutzen:
   ○ gar nicht ○ zeitweise ○ immer

8. Ich habe während des abgelaufenen Tages versucht den Geschmack und/oder die Inhalierbarkeit des Dampfes durch Veränderungen der Einstellungen an der E-Zigarette zu verbessern:
   ○ gar nicht ○ nur wenige Male ○ mehrfach

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

Tag 2

Zeitpunkt der Urinabgabe (morgens):_____

Zeitpunkt der Speichelabgabe (morgens):_____

Uhrzeit	Anzahl der Züge	Leistung (in Watt)	Luftstrom (1-4)
0:00 – 1:00			
1:00 – 2:00			
2:00 – 3:00			
3:00 – 4:00			
5:00 – 6:00			
6:00 – 7:00			
7:00 – 8:00			
8:00 – 9:00			
9:00 – 10:00			
10:00 – 11:00			
11:00 – 12:00			
12:00 – 13:00			
13:00 – 14:00			
14:00 – 15:00			
15:00 – 16:00			
16:00 – 17:00			
17:00 – 18:00			

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

18:00 – 19:00			
19:00 – 20:00			
20:00 – 21:00			
21:00 – 22:00			
22:00 – 23:00			
23:00 – 24:00			

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen):
   ○ gut ○ zufriedenstellend ○ ungenügend

2. Die Inhalierbarkeit des erzeugten Dampfes war:
   ○ gut ○ zufriedenstellend ○ ungenügend

3. Ich hatte während des abgelaufenen Tages das Bedürfnis wieder mein eigenes Produkt zu benutzen:
   ○ gar nicht ○ zeitweise ○ immer

4. Ich habe während des abgelaufenen Tages versucht den Geschmack und/oder die Inhalierbarkeit des Dampfes durch Veränderungen der Einstellungen an der E-Zigarette zu verbessern:
   ○ gar nicht ○ nur wenige Male ○ mehrfach

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

Tag 3

Zeitpunkt der Urinabgabe (morgens):_____

Zeitpunkt der Speichelabgabe (morgens):_____

Uhrzeit	Anzahl der Züge	Leistung (in Watt)	Luftstrom (1-4)
0:00 – 1:00			
1:00 – 2:00			
2:00 – 3:00			
3:00 – 4:00			
5:00 – 6:00			
6:00 – 7:00			
7:00 – 8:00			
8:00 – 9:00			
9:00 – 10:00			
10:00 – 11:00			
11:00 – 12:00			
12:00 – 13:00			
13:00 – 14:00			
14:00 – 15:00			
15:00 – 16:00			
16:00 – 17:00			
17:00 – 18:00			

Protokoll
Part B

ABF GmbH ANALYTISCH-BIOLOGISCHES FORSCHUNGSLABOR MÜNCHEN

Datum:	Probandennummer:

18:00 – 19:00			
19:00 – 20:00			
20:00 – 21:00			
21:00 – 22:00			
22:00 – 23:00			
23:00 – 24:00			

1. Den Geschmack des mittels E-Zigarette erzeugten Dampfes bewerte ich mit (bitte ankreuzen):
   ○ gut ○ zufriedenstellend ○ ungenügend

2. Die Inhalierbarkeit des erzeugten Dampfes war:
   ○ gut ○ zufriedenstellend ○ ungenügend

3. Ich hatte während des abgelaufenen Tages das Bedürfnis wieder mein eigenes Produkt zu benutzen:
   ○ gar nicht ○ zeitweise ○ immer

4. Ich habe während des abgelaufenen Tages versucht den Geschmack und/oder die Inhalierbarkeit des Dampfes durch Veränderungen der Einstellungen an der E-Zigarette zu verbessern:
   ○ gar nicht ○ nur wenige Male ○ mehrfach

Adverse effects and Deviations

Table 29: Adverse events during the clinical study (ordered by subject number)

Subject	Date	Event	Action taken	Relationship to investigational product	Outcome
101	14.11.2016	Flatulence	None	No	Resolved
101	21.11.2016	Shoulder pain	None	No	Resolved
101	30.11.2016	Heratburn	Concomitant Medication	Yes	Resolved
101	2.12.2016	Headache	None	No	Resolved
102	15.11.2016	Headache	Concomitant Medication	No	Resolved
102	21.11.2016	Headache	None	No	Resolved
103	19.11.2016	Headpressure	None	Yes	Resolved
104	19.11.2016	Dizziness after second vaping session	Water, supine position with feet upright	Yes	Resolved
105	19.11.2016	Backache	None	No	Resolved
105	20.11.2016	Headache	None	No	Resolved
107	4.12.2016	Pressure sensitivity left bend of elbow	None	No	Resolved
109	3.12.2016	Subject fainted during the application of an intravenous vein catheter	Supervision of the blood pressure	No	Resolved
110	15.12.2016	Groin strain	None	No	Resolved
114	6.01.2017	Headache	Concomitant Medication	No	Resolved
114	9.01.2017	Thrombophlebitis right elbow	Concomitant Medication	related to study procedure	Resolved
115	9.01.2017	Common cold	None	No	Ongoing
115	13.01.2017	Headache	None	No	Ongoing
116	14.01.2017	Thrombophlebitis left elbow	None	related to study procedure	Resolved
117	11.01.2017	Headache	None	No	Resolved
117	13.01.2017	Headache	Concomitant Medication	No	Resolved
118	13.01.2017	Orthostatic hypotonic		No	Resolved
118	13.01.2017	Headache	Concomitant Medication	No	Resolved
119	17.02.2017	Nausea	None	No	Resolved
119	17.02.2017	Headache	None	No	Resolved
122	11.11.2016	Headache	None	No	Resolved
123	12.11.2016	Backache	None	No	Resolved
125	12.11.2016	Headache	None	No	Resolved

Table 30: Deviations to the study protocol

Note to file number	Date	Category	Topic	Description
1	13.11.2016	Protocol-Deviation	Subject 123 blood sample missing	due to difficult venipuncture blood sample no. 25 was not collected
2	21.11.2016	Protocol-Deviation	Freezer temperature alarm	decreasing the freezer temperature from -30°C to 35 °C to avoid regular alarms; approved by ABF
3	13.11.2016	Protocol-Deviation	urine sample not assigned to subject	urine sample no. 26 of subject 121 not documented; however subject 124 has an additional urine sample documented; an exact assignment of the sample is not possible
4	1.12.2016	Process related	Sputum-collection	two attempts for sputum collection are enough; missing sputum sample will be documented in the source documents
5	5.12.2016	Process related	Subject 107, urine sampling, unclear documentation	related to U17 – U19: all urine container were shipped to ABF; U17 is not documented in the source documents whereas U19 was documented twice; to clarify the container were weighed again
6	7.01.2017	Administrative	Subject 116, urine sampling, unclear documentation	related to U16: wrong container order; container U16 was used after U12
7	14.03.2017	Protocol-Deviation	Subject 13,19 and 20 – increased BMI	subjects were enrolled with BMI between 29 and 30; approved by ABF
8	4.02.2017	Process related	Day 1 Vaping Session not performed with fresh liquid an unused coil	related to subject 119 and 120: the liquid in the tanks flowed out over night; tanks of subject 115 and 116 were used instead
-	20.11.2016	Process related	Subject 102, urine sampling, missing documentation	Sample U18 present but not documented in source data

Raw data overview Part A and B

Table 31: Results for the three subgroups in Part A of the clinical study, mean ± SD / Median (range)

	Vapers, 10 W	Vapers 18 W[b]	Cigarette Smokers[c]
N	10	10	5
E-liquid consumption (mL/10 sessions)	1.26 ± 0.29/1.34 (0.74 – 1.66)	1.56 ± 0.95/1.33 (0.60 – 3.11)	-
Plasma labeled nicotine peak (ng/ml)	1.38 ± 0.70/1.28 (0.60 – 3.17)	1.90 ± 1.70/1.22 (0.73 – 5.47)	0.96 ± 0.27/0.92 (0.63 – 1.33)
Plasma nicotine peak (ng/mL)	14.39 ± 10.80/11.50 (6.22 – 44.05)	17.68 ± 16.51/10.27 (3.48 – 52.94)	35.07 ± 16.01/25.35 (23.42 – 60.25)
Total Plasma nicotine peak (ng/ml)	13.81 ± 6.97/12.82 (5.96 – 31.67)	18.97 ± 16.96 /12.16 (4.25 – 54.66)	29.95 ±6.64/25.59 (9.02 – 14.32)
AUC labeled plasma nicotine (ng/mL x h)	0.73 ± 0.56/0.32 (0.36 – 2.29)	0.89 ± 0.88/0.52 (0.20 – 2.91)	0.44 ± 0.11/0.43 (0.30 – 0.60)
AUC unlabeled plasma nicotine (ng/mL x h)	7.05 ± 5.61/5.46 (3.80 – 22.78)	8.45 ± 8.21/5.17 (1.93 – 27.41)	12.02 ± 2.95/10.47 (8.99 – 15.53)
Calculated AUC total plasma nicotine (ng/mL x h)[a]	7.27 ± 5.63/5.61 (3.61 – 22.93)	8.89 ± 8.75/5.16 (2.04 – 29.12)	12.44 ± 3.01/12.10 (8.45 – 16.81)
AUC labeled plasma cotinine (ng/mL x h)	3.46 ± 1.28/3.52 (1.47 – 5.58)	4.92 ± 3.37/3.56 (1.83 – 9.63)	3.02 ± 0.83/2.73 (2.32 – 4.32)
Calculated AUC total plasma cotinine (ng/mL x h)[a]	34.57 ±12.75/35.18 (14.74 – 55.79)	49.16 ± 33.71/35.59 (18.28 – 96.29)	84.48 ± 23.35/76.49 (64.84 – 120.98)
AUC labeled saliva cotinine (ng/mL x h)	2.39 ± 0.97/2.41 (0.86 – 4.27)	3.14 ± 2.41/2.11 (0.72 – 8.05)	1.72 ±0.63/1.80 (1.02 – 2.55)
Calculated AUC total saliva cotinine (ng/mL x h)[a]	23.89 ± 9.74/24.07 (8.61 – 42.74)	31.39 ± 24.14/21.09 (7.16 – 80.49)	48.18 ± 17.51/18.01 (28.59 – 71.32)
AUC labeled saliva OH-Cot (ng/mL x h)	0.43 ± 0.30/0.43 (0.00 – 0.89)	0.42 ± 0.27/0.29 (0.16 – 0.83)	0.31 ± 0.04/0.32 (0.27 – 0.37)
Calculated AUC total saliva OH-cot (ng/mL x h)[a]	4.34 ± 3.03/4.27 (0.00 - 8.89)	4.22 ± 2.71/2.87 (1.64 – 8.29)	8.74 ± 1.20/9.04 (7.51 – 10.37)
Excretion of labeled TNE, 0 – 72 h (mg)	0.86 ± 0.42/0.79 (0.31 – 1.55)	0.93 ± 0.70/0.59 (0.33 – 2.30)	0.48 ±0.13/0.42 (0.36 – 0.70)
Calculated excretion of total TNE, 0 – 72 h (mg)[a]	8.56 ± 4.16/7.90 (3.09 – 15.54)	9.35 ± 7.05/5.89 (3.25 – 23.03)	13.50 ± 3.73/11.83 (9.96 – 19.51)
AUC labeled plasma PG (ng/mL x h)	76.55 ± 19.08/81.88 (43.33 – 104.38)	92.85 ± 77.81/55.67 (18.49 – 199.79)	34.22 ± 5.79/32.35 (27.45 – 41.05)
Calculated AUC total plasma PG (µg/mL x h)[a]	0.77 ± 0.19/0.82 (0.43 – 1.04)	0.93 ± 0.78/0.56 (0.18 – 2.00)	0.96 ± 0.16/0.91 (0.77 – 1.15)
Excretion of labeled PG, 0 – 72 h (mg)	2.94 ± 0.52/2.97 (1.95 – 3.88)	4.16 ± 3.51/2.70 (0.85 – 10.47)	1.23 ± 0.29/1.33 (0.78 – 1.51)
Calculated excretion of total PG, 0 – 72 h (mg)[a]	29.40 ± 5.20 (19.52 – 38.79)	41.63 ± 35.16/26.95 (8.46 – 104.73)	34.40 ± 8.20 (21.82 – 42.38)

[a]: 'Total' comprises labeled + unlabeled biomarkers and is calculated by multiplying the value for the labeled analyte with 10 for vapers (based on the ratio in the e-liquid). For cigarette smokers, the nicotine-derived biomarkers were multiplied with 28 (ratio based on AUC for plasma nicotine of smokers: unlabeled + labeled/labeled)
[b]: Significance level for difference between vapers, 10 W and vapers, 18 W: *: $p<0.05$
[c]: Significance level for difference between all vapers (10 W and 18 W) and smokers: +: $p<0.05$

Table 32: Results for 19 vapers in Part B of the clinical study, mean ± SD/Median (range)

		Day -1	Day 1	Day 2	Day 3
E-Liquid consumption (g/d)		-	1.81 ± 1.33/1.76 (0.08 – 4.54)	1.69 ± 1.35/1.88 (0.19 – 4.65)	1.58 ± 1.29/1.56 (0.09 – 4.31)
Wattage set up (W)		-	10.93 ± 4.00/10.00 (6.33 – 20.00)	10.99 ± 4.06/10.00 (5.00 –20.00)	10.62 ± 3.72/10.00 (5.00 – 20.00)
Labeled plasma nicotine (ng/mL)		0.0	0.96 ± 0.98/0.71 (0.00 – 3.81)	0.84 ± 0.92/0.43 (0 – 2.99)	0.90 ± 1.00/0.49 (0.00 – 3.44)
Unlabeled plasma nicotine (ng/mL)		14.19 ± 12.07/11.92 (0.00 – 36.95)	15.47 ± 13.26/9.82 (0.38 – 38.33)	13.53 ± 10.91/11.84 (0.32 – 34.51)	14.49 ± 13.47/11.53 (1.42 – 46.44)
Total plasma nicotine (ng/mL)[a]		0.00	9.58 ± 9.78/7.10 (0 – 38.10)	8.43 ± 9.19/4.25 (0.00 – 29.90)	9.04 ± 10.03/4.90 (0 – 34.40)
Labeled plasma cotinine (ng/mL)		0.00	3.34 ± 2.68/3.44 (0.24 – 8.75)	3.75 ± 3.17/2.96 (0.42 – 10.74)	4.16 ± 3.56/3.87 (0.17 – 9.53)
Unlabeled plasma cotinine (ng/mL)		193.84 ± 165.41/162.56 (20.16 – 730.51)	204.69 ± 152.44/193.28 (19.54 – 572.02)	183.99 ± 130.87/142.14 (21.05 – 428.43)	202.67 ± 162.73/183.71 (11.92 – 581.89)
Total plasma cotinine (ng/mL)[a]		0.00	33.43 ± 26.80/34.40 (2.40 – 87.50)	37.52 ± 31.70/29.65 (4.20 – 107.40)	41.46 ± 35.59/38.70 (1.70 – 95.30)
Labeled plasma PG (µg/mL)		0.00	0.11 ± 0.10/0.08 (0.00 – 0.41)	0.11 ± 0.11/0.07 (0.00 – 0.36)	0.14 ± 0.12/0.12 (0.00 – 0.39)
Unlabeled plasma PG (µg/mL)		11.25 ± 18.86/6.47 (0.48 – 80.12)	5.74 ± 6.71/3.18 (0.49 – 28.76)	6.18 ± 6.05/3.89 (0.56 – 21.16)	10.81 ± 18.62/4.22 (1.37 – 82.92)
Total plasma PG (µg/mL)[a]		0.00	1.15 ± 1.05/0.80 (0.00-4.10)	1.10 ± 1.14/0.70 (0.00 – 3.60)	1.41 ± 1.19/1.20 (0.00-3.90)
Labeled TNE (mg/g crea)	Morning	-	0.36 ± 0.94/0.12 (0.01 – 4.20)	0.42 ± 0.43/0.36 (0.02 – 1.88)	0.47 ± 0.47/0.41 (0.03 – 1.95)
	Evening	0.00	0.27 ± 0.24/0.21 (0.02 – 1.01)	0.47 ± 0.48/0.34 (0.04 – 1.81)	0.51 – 0.49/0.43 (0.04 – 1.55)
Unlabeled TNE (mg/g crea)	Morning	-	15.72 ± 27.64/7.16 (1.01 – 119.20)	8.37 ± 7.20/6.28 (0.57 – 24.52)	8.53 ± 5.67/7.72 (0.48 – 21.20)
	Evening	10.00 ± 7.20/7.04 (1.16 – 27.67)	10.16 ± 7.53/8.30 (1.16 – 26.19)	9.50 ± 8.10/6.88 (0.86 – 31.50)	9.87 ± 6.82/8.32 (1.23 – 26.42)
Total TNE (mg/g crea)[a]	Morning	-	3.56 ± 9.40/1.16 (0.06 – 42.05)	4.18 ± 4.32/3.63 (0.17 – 18.84)	4.67 ± 4.75/4.09 (0.27 – 19.53)
	Evening	0.00	2.73 ± 2.37/2.05 (0.23 – 10.05)	4.65 ± 4.82/3.41 (0.38 – 18.09)	5.12 ± 4.87/4.27 (0.41 – 15.47)
Labeled cotinine in saliva (ng/mL)	Morning	-	1.35 ± 1.22/1.13 (0.09 – 3.87)	2.61 ± 2.32/2.86 (0.21 – 9.14)	2.51 ± 2.88/0.97 (0.00 – 9.48)
	Evening	0.00	1.72 ± 1.32/1.68 (0.17 – 4.62)	2.02 ± 1.86/1.10 (0.02 – 6.09)	2.60 ± 2.36/2.20 (0.11 – 7.50)
Unlabeled cotinine in saliva (ng/mL)	Morning	-	137.83 ± 105.77/111.98 (13.83 – 379.24)	132.67 ± 108.29/108.99 (8.00 – 348.62)	121.81 ± 105.81/81.19 (0.07 – 319.61)
	Evening	131.41 ±107.05/116.80 (8.95 – 385.76)	126.89 ± 92.46/108.59 (10.52 – 322.71)	123.53 ± 102.69/104.59 (7.52 – 408.89)	131.32 ± 93.27/119.58 (6.91 – 330.22)
Total cotinine in saliva (ng/mL)[a]	Morning	-	13.52 ± 12.22/11.30 (0.90 – 38.70)	26.09 ± 23.17/28.60 (2.10 – 91.40)	25.06 ± 28.79/9.70 (0.00 – 94.80)
	Evening	0.00	17.23 ± 13.16/16.80 (1.70 – 46.20)	20.15 ± 18.62/11.00 (0.20 – 60.90)	25.97 ± 23.65/22.00 (1.10 – 75.00)
Labeled OH-cot in saliva (ng/mL)	Morning	-	0.27 ± 0.36/0.14 (0.00 – 1.56)	0.63 ± 0.84/0.39 (0.00 – 3.61)	0.64 ± 0.91/0.22 (0.00 – 3.77)
	Evening	0.00	0.29 ± 0.27/0.19 (0.00 – 0.85)	0.48 ± 0.55/0.22 (0.00 – 2.17)	0.63 ± 0.68/0.29 (0.00 – 2.39)
Unlabeled OH-cot in saliva (ng/mL)	Morning	-	22.06 ± 18.95/14.77 (0.20 – 76.54)	20.96 ± 18.95/18.56 (0.25 – 73.13)	19.71 ± 18.49/16.78 (0.00 – 78.62)
	Evening	19.35 ± 13.27/17.99 (0.23 – 53.03)	18.08 ± 13.43/15.93 (0.27 – 48.94)	19.19 ± 15.82/17.16 (0.24 – 53.81)	22.11 ± 16.75/19.79 (0.20 – 62.10)
Total OH-cot in saliva (ng/mL)[a]	Morning	-	2.72 ± 3.64/1.40 (0.00 – 15.60)	6.28 ± 8.44/3.90 (0.00 – 36.10)	6.39 ± 9.08/2.20 (0.00 – 37.70)
	Evening	0.00	2.86 ± 2.71/1.90 (0.00 – 8.50)	4.77 ± 5.53/2.20 (0.00 – 21.70)	6.31 ± 6.83/2.90 (0.00 – 23.90)

Table 32 Continuation: Results for 19 vapers in Part B of the clinical study, mean ± SD/Median (range)

Labeled PG (mg/g crea)	Morning	-	2.48 ± 6.77/0.82 (0.08 – 30.26)	1.70 ± 2.29/0.89 (0.00 – 9.80)	1.19 ± 1.52/0.75 (0.00 – 5.10)
	Evening	0.00	1.95 ± 1.57/1.31 (0.28 – 5.87)	2.11 ± 2.17/1.48 (0.10 – 8.50)	1.87 ± 1.66/1.10 (0.00 – 4.72)
Unlabeled PG (mg/g crea)	Morning	-	57.66 ± 120.52/13.54 (1.20 – 496.98)	158.07 ± 534.55/14.12 (0.52 – 2344.21)	68.39 ± 167.62/17.64 (1.84 – 704.84)
	Evening	116.20 ± 276.42/40.25 (1.24 – 1236.53)	56.47 ± 67.72/37.05 (4.83 – 292.34)	72.70 ± 102.15/35.26 (3.84 – 404.20)	112.20 ± 207.47/34.53 (3.24 – 863.32)
Total PG (mg/g crea)[a]	Morning	-	24.80 ± 67.69/8.17 (0.84 – 302.62)	16.99 ± 22.92/8.94 (0.00 – 98.03)	11.94 ± 15.17/7.51 (0.00 – 50.89)
	Evening	0.00	19.52 ± 15.70/13.14 (2.76 – 58.72)	21.05 ± 21.68/14.77 (0.96 – 84.99)	18.70 ±16.61/11.04 (0.00 – 47.17)

[a]: 'Total' comprises labeled + unlabeled biomarkers and is calculated by multiplying the value for the labeled analyte with 10 for vapers (based on the ratio in the e-liquid). For cigarette smokers, the nicotine-derived biomarkers were multiplied with 28 (ratio based on AUC for plasma nicotine of smokers: unlabeled + labeled/labeled)

Isotopic correction

Table 33: Correction of the contribution of ^{12}C-isotopes to the ^{13}C-labeled metabolites using the isotopic distribution calculator in Analyst 1.6.3 (SCIEX, Darmstadt, Germany) and the experimental value derived from the calibration curve with native reference standards. The analytes are grouped by method.

	Fragment	Isotopic distribution calculator (theoretical) [%]	Experimental [%]
Nicotine-d_7 in nicotine	C_9H_9N	0.0	0.0
Cotinine-d_7 in cotinine	C_5H_5N	0.0	0.0
OH-cotinine-d_7 in OH-cotinine	C_5H_5N	0.0	0.0
Nornicotine-d_4 in nornicotine	C_9H_8N	0.0	0.0
Norcotinine-d_4 in norcotinine	C_5H_5N	0.0	0.0
Nicotine-N-oxide-d_7 in nicotine-N-oxide	C_9H_7N	0.0	0.0
Cotinine-N-oxide-d_7 in cotinine-N-oxide	C_5H_5N	0.0	0.0
Nicotine-N-glucuronide-d_7 in nicotine-N-glucuronide	$C_{10}H_{14}N_2$	0.0	0.0
Cotinine-N-glucuronide-d_7 in cotinine-N-glucuronide	$C_{10}H_{12}N_2O$	0.0	0.0
OH-cotinine-N-glucuronide-d_7 in OH-cotinine-N-glucuronide	$C_{10}H_{12}N_2O_2$	0.0	0.0
Hydroxy-(pyridyl)-butanoic acid-d_4 in hydroxy-(pyridyl)-butanoic acid	C_7H_9N	0.0	0.0
^{13}C-TCA in TCA	$C_7H_{12}NO_2S$	9.1	8.8
$^{13}C_2$-MTCA in MTCA	$C_8H_{14}NO_2S$	5.3	5.4
^{13}C-TCG in TCG	$C_7H_{12}NO_2S$	9.1	9.0
$^{13}C_2$-MTCG in MTCG	$C_8H_{14}NO_2S$	5.3	5.3
^{13}C-formaldehyde-DNPH in formaldehyde-DMPH	$C_7H_5N_3O_2$	9.0	8.6
$^{13}C_2$-acetaldehyde-DNPH in acetaldehyde-DNPH	$C_8H_8N_4O_4$	1.3	0.3
$^{13}C_3$-acetone-DNPH in acetone-DNPH	$C_9H_{10}N_4O_4$	0.1	0.5
$^{13}C_3$-acrolein-DNPH in acrolein-DNPH	$C_9H_8N_4O_4$	0.1	0.3
$^{13}C_3$-propionaldehyde-DNPH in propionaldehyde-DNPH	$C_9H_{10}N_4O_4$	0.1	0.3

$^{13}C_2$-crotonaldehyde-DNPH in crotonaldehyde-DNPH	$C_{10}H_{10}N_4O_4$	1.5	1.0
$^{13}C_2$-crotonaldehyde-DNPH in crotonaldehyde-DNPH	$C_{10}H_{10}N_4O_4$	0.0	0.1
$^{13}C_2$-methacrolein-DNPH in methacrolein-DNPH	$C_{10}H_{10}N_4O_4$	1.5	0.8
$^{13}C_2$-methacrolein-DNPH in methacrolein-DNPH	$C_{10}H_{10}N_4O_4$	0.0	0.0
NAB-d_4 in NAB	$C_{10}H_{13}N_2$	0.0	0.0
NAT-d_4 in NAT	$C_{10}H_{11}N_2$	0.0	0.0
NNAL-d_7 in NNAL	$C_{10}H_{14}NO$	0.0	0.0
NNK-d_7 in NNK	$C_7H_7N_0$	0.0	0.0
NNN-d_4 in NNN	$C_9H_{11}N_2$	0.0	0.0
Derivatized $^{13}C_3$-PG in derivatized PG	$C_{10}H_{10}O_2$	0.0	0.0
Derivatized $^{13}C_3$-G in derivatized G	$C_{17}H_{14}O_4$	0.0	0.0
$^{13}C_2$-HEMA in HEMA	C_2H_4OS	4.2	5.6
$^{13}C_2$-HMPMA in HMPMA	C_4H_8OS	4.7	6.5
$^{13}C_4$-HMPMA in HMPMA	C_4H_8OS	0.0	0.0
$^{13}C_3$-2-HPMA in 2-HPMA	C_3H_6OS	0.1	0.4
$^{13}C_3$-3-HPMA in 3-HPMA	C_3H_6OS	0.1	0.2
$^{13}C_3$-DHPMA in DHPMA	$C_3H_6O_2S$	0.1	0.2