VRIJE UNIVERSITEIT BRUSSEL

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CHEMICAL AND TOXICOLOGICAL ASSESSMENT OF E-CIGARETTE LIQUIDS

SOPHIA BARHDADI

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Promotors VUB:
Prof. Dr. Ir. Tamara Vanhaecke
Em. Prof. Dr. Apr. Vera Rogiers

Promotor Sciensano:
Dr. Apr. Eric Deconinck

Members of the jury

External experts

Dr. Wouter Visser
Centre for Health Protection
National Institute for Public Health and the Environment (RIVM)

Prof. Adrian Covaci
Department of Pharmaceutical Sciences
Universiteit Antwerpen

Internal members

Prof. Ann Van Eeckhaut
Department of Pharmaceutical and Pharmacological Sciences
Vrije Universiteit Brussel

Prof. Marc Elskens
Department of Chemistry
Vrije Universiteit Brussel

Chairwoman

Prof. Kristien de Paepe
Department of In Vitro Toxicology and Dermato-Cosmetology
Vrije Universiteit Brussel

Promotors

Prof. Tamara Vanhaecke
Department of In Vitro Toxicology and Dermato-Cosmetology
Vrije Universiteit Brussel

Em. Prof. Vera Rogiers
Department of In Vitro Toxicology and Dermato-Cosmetology
Vrije Universiteit Brussel

Dr. Eric Deconinck
Medicines and Health Products
Sciensano

ABSTRACT

The popularity of the electronic cigarette (e-cigarette) has increased significantly in the past decade. In Belgium, the implementation of the revised European Tobacco Products Directive (TPD) in 2014 marked a turning point for this phenomenon. Prior to this, the use of the e-cigarette was not yet mainstream, as only nicotine-free e-cigarettes were allowed on the market. However, as a result of the legislative changes, nicotine-containing e-cigarettes became freely available on the market and 'vapeshops' skyrocketed since then. As the number of e-cigarette users increased, so did the media coverage about the benefits and dangers of the e-cigarette, whether scientifically substantiated or not. To assure public health, scientific research about the safety and quality of the e-cigarette is of great importance. The objective of this PhD thesis was therefore to first determine the chemical composition of the liquids used in e-cigarettes (e-liquid) and next to investigate toxicological aspects of flavouring substances present in e-liquid refills.

First, a comprehensive literature search was performed to obtain an overview about the chemical composition of the e-liquids and the analytical methods used for their detection. Although the analytical methods used in these studies have not always been well validated and thus the results of these studies need to be critically examined; three main problems could be uncovered: (1) the content of nicotine in the e-liquids often does not correspond to the claimed concentration, (2) there is a significant presence of hazardous impurities and contaminants in the e-liquids and (3) food flavourings (diacetyl and acetylpropionyl) which are toxic when inhaled are also present.

In order to analyse the composition of the e-cigarettes and to check the e-liquids for the abovementioned issues, alternative methods were developed for the quantitative determination of nicotine and its related impurities (HPLC-DAD) and for the flavours diacetyl and acetylpropionyl (HS/GC-MS). Also, screening methods were developed for the identification of volatile organic compounds (HS/GC-MS) and the additives taurine (LC-MS/MS) and caffeine (GC-MS). Subsequently, the influence of the revised TPD on the quality of e-liquids available on the Belgian market was investigated using these developed methods. A total of 246 e-liquids were purchased before (2013-2016), during (2016) and after (2017-2018) the implementation of the revised TPD. The samples were examined for the presence of nicotine, nicotine-related impurities, volatile organic compounds, caffeine, taurine and the harmful flavours diacetyl and acetylpropionyl. In general, the legislative changes have had a positive effect on the quality of e-liquids: the results of our study show that the quality of e-liquids has improved following the implementation of the revised TPD. Indeed, in recent years, there have been significantly fewer discrepancies between the effective nicotine content and the claimed concentration. No hazardous volatile organic compounds were found in the 2017-2018

samples compared to the samples before the TPD. In 2018, 5% of the samples contained caffeine, compared to 16% in 2017. The food flavours diacetyl and acetylpropionyl were still present in e-liquids with a sweet, buttery taste such as cake, caramel, popcorn (55% in 2017 compared to 27% in 2018).

Next, as a test case, the risk of inhaling diacetyl present in e-liquids was investigated. An adapted risk assessment methodology for intentional inhalation of substances through the e-cigarette was applied. This exercise showed that there is no risk for systemic toxicity related to diacetyl vapours. However, the risk for local lung toxicity (lung tissue lesions associated with chronic pulmonary bronchiolitis obliterans) could not be excluded in case of repeated exposure to diacetyl through e-cigarette use.

In the final experimental part of the thesis, we focused on the identification of potential genotoxic flavouring substances in e-liquids through the use of non-animal methodologies. As such, 807 flavouring substances were identified in 129 e-liquids using complementary HS-GC MS methods. In a first step, all these substances were screened for genotoxicity using qualitative and quantitative *in silico* models. In total, potential genotoxicity activity was predicted for 44 flavourings. Based on information from European databases, genotoxicity could be confirmed for five of these flavourings (estragole, safrole, 2-furylmethylketone, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone and transhexanal). Genotoxicity could be excluded for 23 flavourings. For the remaining 16 flavourings, insufficient information on their genotoxicity was present. For four of these flavourings, a commercial standard was available and thus could be tested *in vitro* using an Ames- and micronucleus test. One of the four substances was only slightly positive in the micronucleus test (β-hellandrene), while for isoledene, 2,3-butanedione and 2,3-pentanedione a clear positive result was obtained in at least one of the two *in vitro* tests.

Finally, in order to minimise potential health risks imposed by the use of e-cigarettes, some recommendations are suggested to further amend the current e-cigarette legislation.

SAMENVATTING

De populariteit van de elektronische sigaret (e-sigaret) is de laatste jaren in een mum van tijd gestegen. In België heeft het in voege treden van de herziene Europese Tabaksproductenrichtlijn (Tobacco Product Directive ofwel TPD) in 2014 voor een keerpunt gezorgd. Vóór deze datum was het gebruik van de e-sigaret nog niet alomtegenwoordig, omdat enkel nicotinevrije e-sigaretten beschikbaar waren, en dit uitsluitend in de apotheek. Na de herziening van de TPD kwamen nicotine bevattende e-sigaretten echter ook beschikbaar op de markt en zag men het aantal 'dampwinkels' als paddenstoelen uit de grond schieten. Met het aantal toenemende e-sigaret gebruikers, steeg ook de media aandacht over de voordelen en de gevaren van de e-sigaret, al dan niet wetenschappelijk onderbouwd. Wetenschappelijk onderzoek naar de veiligheid en kwaliteit van de e-sigaret is daarom van groot belang voor de algemene volksgezondheid. De doelstelling van dit proefschrift was daarom om eerst de chemische samenstelling te bepalen van de vloeistof die gebruikt wordt in e-sigaretten (e-vloeistof) en vervolgens te focussen op toxicologische aspecten van smaakstoffen aanwezig in e-vloeistoffen.

Er werd eerst een uitvoerig literatuuronderzoek gedaan met betrekking tot de chemische samenstelling van e-vloeistoffen en de analytische methoden gebruikt voor hun detectie. Ondanks dat de gebruikte analytische methoden niet altijd gevalideerd waren en de resultaten van deze studies kritisch moeten bekeken worden, konden 3 problemen worden blootgelegd: (1) het gehalte aan nicotine in de e-vloeistoffen komt vaak niet overeen met de geclaimde concentratie, (2) gevaarlijke onzuiverheden en contaminanten zijn significant aanwezig in de e-vloeistoffen en (3) voedingssmaakstoffen (diacetyl en acetylpropionyl) die toxisch zijn bij inhalatie zijn eveneens aanwezig.

Om de samenstelling van de e-sigaretten te analyseren en de e-vloeistoffen te kunnen controleren voor bovenstaande kwesties, werden alternatieve methoden ontwikkeld voor de kwantitatieve bepaling van nicotine en zijn onzuiverheden (HPLC-DAD) en voor de smaakstoffen diacetyl en acetylpropionyl (HS/GC-MS). Er werden ook screeningmethoden ontwikkeld voor de identificatie van vluchtige organische componenten (HS/GC-MS) en de additieven taurine (LC-MS/MS) en cafeïne (GC-MS). Uiteindelijk werd a.d.h.v. de ontwikkelde methoden onderzocht welke invloed de herziene TPD heeft gehad op de kwaliteit van e-vloeistoffen beschikbaar op de Belgische markt. In totaal werden 246 e-vloeistoffen gekocht vóór (2013-2016), tijdens (2016) en na (2017-2018) de implementatie van de herziene TPD. De stalen werden onderzocht op aanwezigheid van nicotine, nicotine gerelateerde onzuiverheden, vluchtige organische stoffen, cafeïne, taurine en de schadelijke smaakstoffen diacetyl en acetylpropionyl. In het algemeen heeft de veranderde wetgeving een positief effect gehad op de

kwaliteit van e-vloeistoffen. De laatste jaren komen er significant minder discrepanties voor tussen het effectieve nicotine gehalte en de geclaimde concentratie. In de stalen van 2017-2018 werden geen gevaarlijke vluchtige organische stoffen teruggevonden tegenover de stalen voor de TPD. In 2018 werd in 5% van de stalen cafeïne teruggevonden, tegenover 16% in 2017. De smaakstoffen diacetyl en acetylpropionyl waren nog steeds aanwezig in e-vloeistoffen met een zoete-, boterachtige smaak zoals de smaak cake, karamel, popcorn (55% in 2017 tegenover 27% in 2018).

Vervolgens werd als test casus onderzocht wat het risico is van inademing van diacetyl aanwezig in e-vloeistoffen. Een aangepaste methodologie van risicobeoordeling voor intentionele inhalatie van substanties via de e-sigaret werd toegepast. Hieruit bleek dat er geen risico is voor systemische toxiciteit als gevolg van het dampen van diacetyl. Echter, het risico op lokale longtoxiciteit (letsels ter hoogte van het longweefsel gelinkt aan de chronische longziekte bronchiolitis obliterans) kon niet uitgesloten worden bij herhaaldelijke blootstelling aan diacetyl via e-sigaret gebruik.

Als laatste experimenteel onderdeel van deze doctoraatsthesis hebben we ons gericht op de identificatie van potentiële genotoxische smaakstoffen in e-vloeistoffen door toepassing van proefdiervrije methoden. A.d.h.v. complementaire HS-GC MS methodes werden er 807 smaakstoffen geïdentificeerd in 129 e-vloeistoffen. Al deze stoffen werden vervolgens in een eerste stap d.m.v. kwalitatieve en kwantitatieve *in silico* modellen *gescreend* voor genotoxiteit. In totaal werd er voor 44 smaakstoffen een potentieel genotoxische activiteit voorspeld. Op basis van informatie uit Europese databanken kon de genotoxiciteit bevestigd worden voor vijf smaakstoffen (estragole, safrole, 2-furylmethylketon, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone en transhexanal). Voor 23 smaakstoffen kon de genotoxciteit uitgesloten worden. Voor de overige 16 smaakstoffen was er onvoldoende informatie over hun genotoxiciteit voorhanden. Voor vier van deze 16 smaakstoffen was een commerciële standaard beschikbaar en konden ze vervolgens *in vitro* getest worden a.d.h.v. een Ames test en een micronucleus test. Eén van de vier geteste stoffen was slechts licht positief in de micronucleustest (β-phellandrene), terwijl voor isoledene, 2,3-butanedione en 2,3-pentanedione in ten minste één van de twee *in vitro* testen een duidelijk positief resultaat werd bekomen.

Teneinde potentiële gevaren voor de gezondheid verbonden aan het gebruik van e-sigaretten tot een minimum te beperken, werden tot slot nog enkele aanbevelingen geformuleerd om de huidige wetgeving inzake e-sigaretten bij te sturen.

TABLE OF CONTENT

LIST OF ABBREVIATIONS

AEMSA	American E-liquid Manufacturing Standards Association
ANFOR	Association Française de Normalisation
ANOVA	Analysis Of Variance
AO	Acridine Orange
AP	Acetylpropionyl
API	Active Pharmaceutical Ingredient
ATR-IR	Attenuated Total Reflectance Infrared Spectroscopy
BaP	Benzo(A)Pyrene
BTEX	Benzene, Toluene Ethylbenzene And Xylene
bw	bodyweight
CAS	Chemical Abstract Service
CBD	Cannabidiol
CBPI	Cytokinesis-Block Proliferations index
CEN	European Committee for Standardization (Comité Européen de Normalisation)
CHO	Chinese Hamster Ovary
CLP	Classification, Labelling And Packaging
CMR	Carcinogenic, Mutagenic Or Toxic To Reproduction
COPD	Chronic Obstructive Pulmonary Disease
D	Delta, Difference
DA	Diacetyl
DAD	Diode Array Detector
DART	Direct Analysis in Real Time
DIY	Do It Yourself
DMSO	Dimethyl Sulfoxide
EC	European Commission
ECHA	European Chemical Agency
EDQM	European Directorate For The Quality Of Medicines And Health Care
EEC	European Economic Community
EFSA	European Food Safety Agency
EI	Electron Impact
EIC	Extracted Ion Chromatogram
ENDS	Electronic Nicotine Delivery Systems
EU	European Union
FAMHP	Federal Agency For Medicines And Health Products
FDA	Food And Drug Administration
FID	Flame Ionization Detector

FRC	Functional Residual Capacity
FWHM	Full Width at Half Maximum
G	Glycerol
GC	Gas Chromatography
GMP	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
HILIC	Hydrophilic Liquid Interaction Chromatography
HPHCs	Harmful and Potential Harmful Constituents
HRMS	High Resolution Mass Spectrometer
HS	Headspace
HS-GC-MS	Headspace Gas Chromatography Mass Spectrometry
HTPs	Heated Tobacco Products
IARC	International Agency For Research on Cancer
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IMS	Ion Mobility Spectroscopy
IR	Infra-Red
IS	Internal Standard
ISO	International Organization for Standardization
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit Of Detection
LOF	Lack Of Fit
LOQ	Limit Of Quantification
m/z	Mass To Charge
MeOH	Methanol
Min	Minutes
MMS	Methyl methanesulfonate
MN	micronuclei
MOE	Margin Of Exposure
MoS	Margin of Safety
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
NA	Not Applicable
NAB	N-Nitrosoanabasine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAT	N’-Nitrosoanatabine

NIR	Near Infrared
NIST	National Institute for Standards and Technology
NMR	Nuclear Magnetic Resonance
NNK	Nicotine-derived nitrosamine ketone
NNN	N-Nitrosonornicotine
NOAEC	No Observable Adverse Effect Concentration
NOAEL	No Observable Adverse Effect Level
NPD	Nitrogen–Phosphorus Detector
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic Aromatic Hydrocarbons
PBPK	Physiologically Based PharmacoKinetic
PBS	Phosphate-buffered saline
PG	Propylene Glycol
Ph.Eur	European Pharmacopoeia
POD	Point Of Departure
ppm	Parts Per Million
PTV	Programmable Temperature Vaporizing
QC	Quality Coefficient
QSAR	Quantitative Structure-Activity Relationship
RD	Royal Decree
REACH	Registration, Evaluation, Authorization And Restriction Of Chemicals
RIVM	Rijksinstituut voor Volksgezondheid en Milieu Ministerie van Volksgezondheid
rpm	Rotation Per Minute
Rs	Resolution
RSD	Relative Standard Deviation
S/N	Signal To Noise
SA	Structural Alert
SD	Standard deviation
SDL	Screening Detection Limit
SED	Systemic Exposure Dose
SIM	Selected Ion Monitoring
SMILES	Simplified Molecular Input Line-Entry System
TC	Temperature Control
THC	Tetrahydrocannabinol
TIC	Total Ion Chromatogram
TOF	Time-of-flight
TPD	Tobacco Product Directive
TSD	Thermionic specific detector

TV	Tidal Volume
TWA	Time-weighted average
UHPLC	Ultra High Pressure Liquid Chromatography
US	United States
USP	United States Pharmacopoeia
UV	Ultraviolet
V	Volt
VIS	Visible Light
VOC	Volatile Organic Compounds
VUB	Vrije Universiteit Brussel
WHO	World Health Organization

OUTLINE

The PhD manuscript starts with a general introduction on e-cigarettes and an overview of the European Tobacco Directive (TPD) (**Chapter I and II),** followed by the data generated during this research project. The latter is subdivided in two major parts; an analytical and a toxicological part. Each part consists of stand-alone chapters based on accepted or submitted peer-reviewed manuscripts.

In **Part I**, the main objective was to generate a general strategy for the chemical characterization of e-liquids. In **Chapter III and IV,** an overview is given of the chemical components detected in e-liquids on the Belgian market and the analytical methods that have been described in literature. The three major problems associated to the quality and safety of e-liquids identified in these review chapters are further discussed in the experimental chapters. A first issue is related to the presence of the controversial flavours diacetyl and acetylpropionyl in e-liquids. In the experimental **Part II** we therefore developed and validated an analytical method to identify and quantify both components (**Chapter V)**. Secondly, issues related to nicotine, more specifically nicotine label discrepancies and the presence of nicotine impurities, are investigated. In **Chapter VI,** the development and validation of the methods used for the analysis of nicotine and its impurities are described. Both quantification methods (together with screening methods for other relevant chemical components) were subsequently used to analyse 246 e-liquid samples available on the Belgian market (**Chapter VII).** This chapter also describes the impact of the TPD revisions implemented in 2016 on the composition and quality of the investigated e-liquids.

In the last, more toxicological oriented **Part III** of the thesis, exposure data obtained through the above mentioned market study has been used to perform a risk assessment related to the exposure of the food flavouring diacetyl through vaping (**Chapter VIII)**. In **Chapter IX**, the genotoxic profile of flavouring components present in e-liquids has been determined.

Finally, to conclude the thesis, all obtained data are reviewed, conclusions are formulated and future perspectives for follow-up research priorities are described.

SCOPE

During the last 10 years, the worldwide use of e-cigarettes has increased rapidly. As such, the latest National Health Survey held in 2018 demonstrated that 15,5% of the Belgian population has tried the e-cigarette.

The revival of nicotine use through alternative tobacco products is, however, associated with the potential introduction of a new public health hazard. Indeed, some scientists claim that the introduction of e-cigarettes will renormalize tobacco use. Additionally, a new lung disease associated with e-cigarette use, namely the E-cigarette or Vaping product use Associated Lung Injury (EVALI) has emerged. On the other hand, the shift from traditional cigarettes to e-cigarettes could also help smokers to quit smoking tobacco and hence cessation of the well-established health risks associated with the latter. Overall, as long as long-term research findings on potential health effects of vaping are not available, the whole picture on e-cigarette use will remain controversial and confusing. Furthermore, there is currently also no agreement on the analytical and toxicological methodologies to be used for the quality and safety assessment of the various chemicals present in e-cigarettes. Even more, the regulatory framework is still developing and is not yet up to date with e-cigarette reality.

Therefore, the scope of this PhD research project was to determine the chemical composition and hence quality of different e-liquids on the Belgian market (Part I and II), and to subsequently link their chemical composition to possible health hazards with focus on flavouring substances (Part III). In order to reach this goal, several research questions have been addressed:

1) Which hazardous components present in e-liquids could pose a potential threat to public health?

At the time of the start of this project, the information on ingredients and other potential hazardous substances present in e-liquids was scarce and only a limited legal framework was defined. Moreover, a lot of controversy existed on the obtained data which were generated by the many different analytical methods. Therefore, a thorough literature search was first conducted to critically review the state of art of the methods described in literature (Chapter IV).

2) Can a general strategy be developed to assess the quality of e-liquids under the current legal framework?

The revised Tobacco Products Directive (TPD) that came into force in 2016 defines, albeit sometimes vaguely, the minimal ingredient requirements to assure the quality and safety of e-cigarette products (Chapter II). In this thesis, (alternative) easy to implement analytical methods to investigate the

validity of the criteria stated in the TPD have been developed and validated to assure the reliability of the obtained results (Chapter V and VI).

3) Did the introduction of the revised TPD in 2016 affect the quality of e-liquids on the Belgian market?

Since this research project started before the implementation of the adopted TPD in 2016, we had the opportunity to assess the impact of the TPD revisions and its implementing acts on e-cigarette constituents and quality on the Belgian market. This has been done by applying the general strategy and methods developed under research question 2 (Chapter VII).

In the last experimental part of the thesis we focused on the toxicological assessment of e-liquid substituents, more specifically flavourings, via e-cigarette use.

4) What is the risk associated with the use of certain flavourings, which are known for their inhalation toxicity?

It is challenging to predict the outcome of the inhalation exposure of chemical substances through vaping. Here, as a case-study, we present a risk assessment for exposure to diacetyl through vaping. Diacetyl, is a well-known flavouring in the food industry, but has also been reported to be toxic via inhalation.

5) Are there any genotoxic flavouring substances present in e-liquids?

The flavourings identified in the investigated e-liquid samples in this thesis were screened for potential genotoxicity. Hereto, only alternative animal-free methods were used including *in silico* tools and *in vitro* toxicity tests (Chapter IX).

To conclude, evidence-based recommendations are suggested to improve/amend the current e-cigarette legislation

PART I: INTRODUCTION

CHAPTER I – INTRODUCTION TO ELECTRONIC CIGARETTES

In 2008, the World Health Organization (WHO) named “tobacco use” as the world's single greatest preventable cause of death [1]. According to the WHO “*the tobacco epidemic is one of the biggest public health threats the world has ever faced, killing more than 8 million people a year around the world. More than 7 million of those deaths are the result of direct tobacco use while around 1.2 million are the result of non-smokers being exposed to second-hand smoke*”. In Belgium, the most recent health survey of 2018 indicates that 15.4% of the population are daily smokers. This is 18% less compared to the previous survey (19% daily smokers in 2013) and a quarter less than 10 years ago (20.5% in 2008) [2].

Smoking is indeed the main cause of the development of lung cancer (90% of cases) and directly responsible for the development of chronic pulmonary diseases such as chronic obstructive pulmonary disease (COPD) and emphysema [3]. Smoking is also a known risk factor for one in three cancers (lung, larynx, oesophagus, stomach,…) [4]. Tobacco use has also been associated with an increased risk of developing cardiovascular diseases (infarct, cerebrovascular accident, high blood pressure, peripheral arterial disease) [5], [6]. Additionally, smokers have a higher risk of developing type 2 diabetes and kidney diseases [7], [8]. Moreover, it is well known that cigarette smoking during pregnancy is a risk factor for low birth weight, premature birth and cot death [9], [10]. Also, environmental tobacco smoke or "passive smoking" is a factor related to lung cancer and cardiovascular disease in non-smokers, as well as respiratory diseases in young children [6], [11].

There are more than 7,000 chemicals present in cigarette smoke [12] of which more than 70 have been linked to cancer [13], [14]. The majority of the chemicals are found in the tar produced by smoking cigarettes. Cigarette tar is a term used to describe the toxic chemical particles left behind by burning tobacco. There are other substances present in tobacco products and cigarette smoke that cause or could cause harm. These components were published by the US Food and Drug Administration (FDA) in a list of 93 Harmful and Potential Harmful Constituents (HPHCs) [15]. This HPHC list focuses on chemicals that are linked to the five most serious health effects of tobacco i.e. cancer, cardiovascular disease, respiratory effects, reproductive problems and addiction. These components are present in tobacco because of the way it is processed (tobacco-specific nitrosamines) or as a result from its combustion (benzopyrines). Tobacco smoke also contains the gas carbon monoxide (CO). This toxic gas is able to displace oxygen from haemoglobin molecules. However, the amount of CO in tobacco smoke is too small to actually lead to hypoxia, because of the increased production of red blood cells as compensation. Tobacco also contains the highly-addictive alkaloid

nicotine which is a stimulant, and in the general population known as the most characteristic constituent of tobacco.

Over the years, the tobacco industry has invented different ways to reduce the yields of tar in cigarettes through the use of filters, filter ventilation, light- and low tar cigarettes, as a way to claim "healthier" cigarettes.

As such, a decade ago, the electronic (e)-cigarette was introduced as an alternative device to inhale nicotine. E-cigarettes are defined by the WHO as " *Electronic nicotine delivery systems (ENDS), of which electronic cigarettes are the most common prototype, are devices that do not burn or use tobacco leaves but instead vaporise a solution the user then inhales. The main constituents of the solution, in addition to nicotine when nicotine is present, are propylene glycol, with or without glycerol and flavouring agents. ENDS solutions and emissions contain other chemicals, some of them considered to be toxicants."* [16]. The e-cigarette is one of the latest inventions to reduce harmful elements present in cigarette smoke [17]. The concept of vaporizing liquid and delivery of the generated aerosol to the user itself was first patented in the 1930s. However, a prototype was never manufactured [18]. In 1965, Herbert A. Gilbert patented a device that closely resembles the modern e-cigarette and manufactured a prototype, which was never commercialized at the time [19]. It was until 2003 that a Chinese pharmacist, Hon Lik, developed the first commercialized e-cigarette under the name *Ruyan*, which means "like smoke" [20]. Finally, in 2006, the first generation of e-cigarettes was introduced in the US and Europe. Since then, the development of different types of e-cigarettes and other ENDS has known a rapid evolution, that is still going on.

1 BASIC COMPONENTS OF E-CIGARETTE DEVICES

The International Organization for Standardization (ISO) has defined general vapour products as "*devices intended for human use, which normally contain electronic components that vaporize a liquid to generate an aerosol carried by the air drawn through the device by the user"* [21]. This definition includes a wide variety of ENDS products such as e-cigarettes, e-cigars, e-shisha and e-pipes. Thus, the main principle of the e-cigarette is that a liquid is heated and consequently vaporized into an aerosol which can be inhaled by the user. As such, the basic design of e-cigarettes consists of the following three components [22]:

(1) a part which holds a liquid solution (e-liquid or e-juice) typically a mixture of propylene glycol and glycerol with nicotine and flavouring chemicals;
(2) a power source, usually a battery;
(3) the heating element (atomizer).

E-cigarette devices can be designed in several ways. They exist either as a single device or as a multiple component product. They can be disposable, rechargeable and/or refillable. In the next paragraph, the various types of e-cigarettes with the different abovementioned characteristics are discussed.

2 EVOLUTION OF E-CIGARETTE DEVICES

The first e-cigarettes available on the market were vapour devices, that resembled the tobacco cigarette in appearance (white body and tan mouthpiece), size and shape (Figure 1.1). Therefore, this first generation is often called *cig-a-likes.* This type of e-cigarette is activated by a power button that must be held during use or by drawing breath through the device which triggers an electronic airflow sensor in the battery section. This type was first disposable, once the battery was discharged. Soon, rechargeable batteries were introduced from an eco-friendly point of view. While the battery is separated from the other compartments, the liquid and heating compartment are combined in a cartomizer which is an association of the cartridge and the atomizer (see section on second generation e-cigarettes). A cartridge is a liquid-soaked poly-foam (sponge-like polyester fibre material) that acts as an e-liquid holder [23]. The heating element consisting of a metal coil is integrated into this liquid chamber. When the e-cigarette is activated, the resistance wire coil heats up and vaporizes the liquid around it [24]. The pre-filled cartomizer is discarded when empty and all the liquid is vaporized. The user notices this as the vapour starts to taste burnt as a result of heating insufficient liquid with a dry coil. Refillable e-liquid cartomizers also exist.

The second generation e-cigarette is characterized by its larger size and cylindrical shape, more resembling a large pen instead of a tobacco cigarette. These devices are also called *tank-systems* because of the reservoir that can hold larger volumes of e-liquid than the first generation cartridge-containing models. This tank or clearomizer is the storage space for the liquid, which can be refilled and also contains an atomizer head which is replaceable. An atomizer is the part that contains the heating coil and a wick part which is saturated with e-liquid and transfers the e-liquid to the coil.

The third generation e-cigarette devices include a diverse set of product designs (cylindrical or box-shaped), all characterized by a large-capacity lithium battery that allows the user to adjust the voltage and/or wattage delivered to the atomizer (Figure 1.1). These batteries can be combined either with second generation atomizers or with customizable atomizers where the user can adjust himself the type of coil and wick. Hence, these third generation ENDS devices are called *mods* (referring to modifications) or *Advanced Personal Vaporizers (AVPs*). These newer devices also produce more aerosol than the previous generations and thus have an even greater influence on the levels of chemicals (*e.g.* nicotine) delivered to the user [25].

The most recent, fourth generation e-cigarette devices are basically third generation devices with extra temperature control, meaning that the user can set a temperature limit. The vaporization temperature thus stays stable with longer and more frequent puffs, thereby reducing the risk for dry and burnt hits[1] [26].

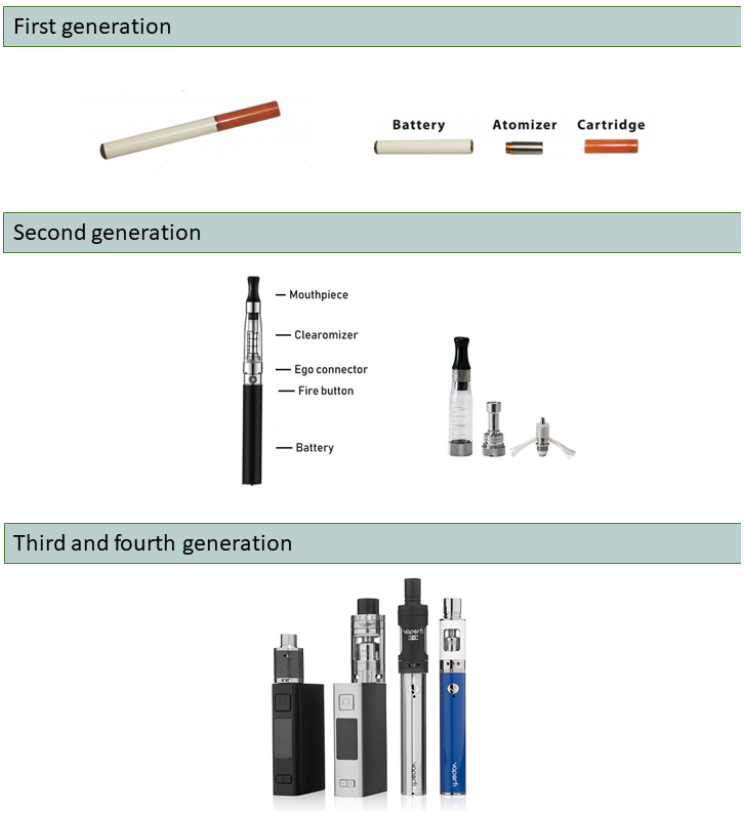


***Figure 1.1**: Different generations of e-cigarette devices.*

3 E-CIGARETTE OPERATION AND DETAILED DEVICE COMPONENTS

E-cigarette products evolve rapidly, introducing high performance variability between the different e-cigarette devices. For example, the first generation devices have a low aerosol production and low nicotine delivery potential compared to the newer devices that have a nicotine delivery equivalent to tobacco cigarettes [25]. Furthermore, these design variations might as well lead to differences in potential health risks including the presence of (potentially) harmful constituents in emissions of the aerosols [27]. Therefore, it is important to take a closer look at the different stages of the operation of the e-cigarette device that could impact the generation of the aerosol.

[1] Dry puffs or burnt puffs are puffs with unpleasant taste due to liquid overheating in the atomizer.

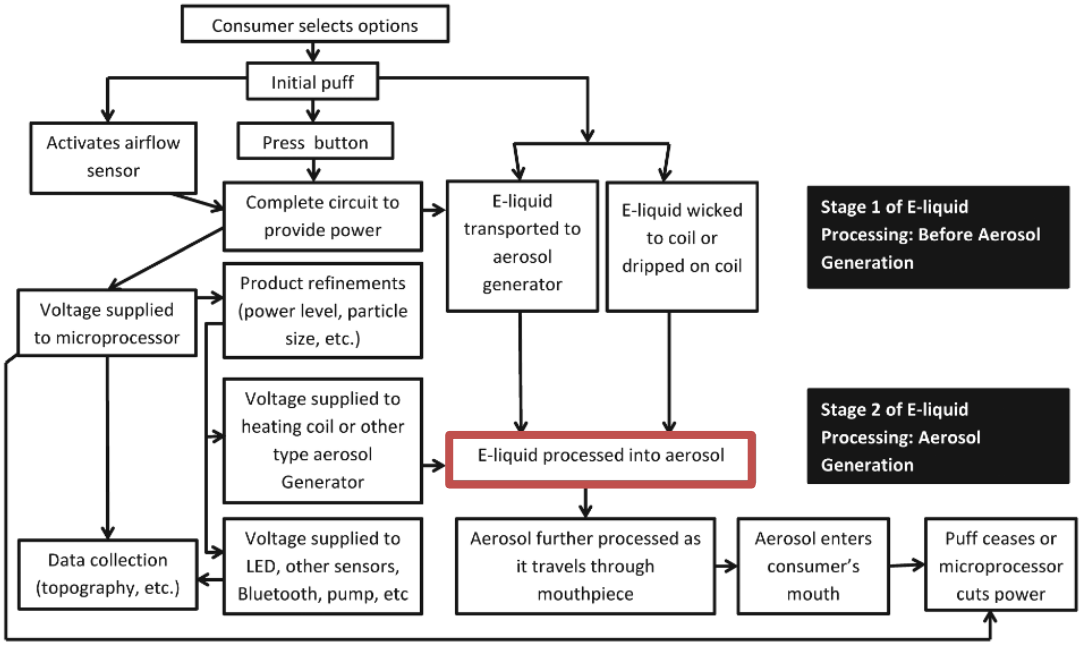


***Figure 1.2**: Basic e-cigarette operation to transform and deliver e-liquid-based aerosol [22].*

The key event of the whole operation of the e-cigarette is the aerosol production. The actions and functions before, during and after the generation of the aerosol are given in the flowchart depicted in Figure 1.2.

Stage 1 – E-liquid processing before aerosol generation:

Prior to the production of the aerosol, the e-liquid needs to be transferred to the aerosol generator (= heating element). The heating element will heat up the liquid that is presented in the wicking material. Wicking material are heat-resistant absorbent materials. Most common used wicking materials are cotton, silica and oxidized stainless steel mesh. Less common are bamboo and hemp. The most important function of the wicking material is to retain the liquid so it can be transferred to the heating coil which is wrapped around the wicking material. In the first generation e-cigarettes, the cig-a-likes, the wicking material also helps to contain the liquid in the cartomizer, to prevent leaks. In theory, the materials are heat-resistant, though contaminants from wicking materials in aerosol emissions are not uncommon. The wick also controls the delivery of e-liquid to the heating element through its capillary forces. In the case there is insufficient e-liquid to be transferred through the wick, the heating element will reach extremely high temperature. This will cause the wicking material which is wrapped around the heating element, to burn which results in an unpleasant, burnt odour and taste of the aerosol (= dry puff) [28]. The liquid saturation rate of the wicking material is important to prevent dry puffing. Depending on the place of the heating element in a liquid tank, bottom or top, the liquid-wick rates are variable, making them less or more prone to dry puffs. Top coil tanks, with the heating element

on top of the liquid reservoir, require longer wicks. These are therefore more prone to dry puffs because of the poor liquid/wick rate, which also means that the maximum power that can be applied on the heating element is limited. Bottom coil tanks, with the heating element in the bottom of the liquid reservoir, require very short wicks, because the e-liquid transfer to the wick relies also on gravity. These devices are more appropriate for high power battery devices, as these are less prone to dry puffing [29]. Finally, a small group of users manually adds or drips the liquid directly onto the wick and coil. This mode of e-cigarette use is highly prone to dry puffs and therefore not recommended [30].

Stage 2 - Aerosol generation:

The second stage of aerosol processing involves the actual aerosol generation due to heating of the e-liquid through contact with the heating element (Figure 1.3). Heating the e-liquid requires a voltage supplied to a metal resistance wire. The heating process in e-cigarettes is based on the Joule effect, also known as resistive heating [22]. Whenever an electric current is applied on a material that has some resistance, it creates heat. Thus, an electric current passing through a resistor will convert that electrical energy into heat energy. Thus, the heating power of an e-cigarette depends on a combination of the resistance value of the heating filament (= coil) used and the voltage applied (= battery). Heat is created through the Joule effect, and the power of heat can be calculated by Joule's law that states that the power of heating, generated by an electrical conductor, is proportional to the product of its resistance and the square of the current [31]:

$$P = IV = I^2R = V^2/R$$

P = the power (energy per unit time) converted from electrical energy to thermal energy;
I = the current travelling through the resistor or other element;
R = the resistance;
V = the electric potential or voltage.

The heating element is the key component as it converts electrical energy into heat through the process of Joule heating. As mentioned before this heating element is a metal resistant filament called a coil. The most common materials used are Nichrome and Kanthal, because their resistance stays relatively constant with changing temperature. Most heating elements use a Nichrome 80/20 (80% nickel, 20% chromium) wire which is an ideal material, because it has relatively high resistance. Additionally, when it is heated for the first time, it forms a layer of chromium oxide, which prevents the wire to break or to burn out as the material beneath the chromium oxide layer will not oxidize.

Because of this high corrosion resistance, Nichrome withstands temperatures up to 1150°C [32]. Kanthal is an iron-chromium-aluminium alloy with a high resistance and good corrosion resistance at high temperatures. Behind the Kanthal name a letter or number / letter combination is present. Kanthal A1 or Kanthal D are usually used for coils. The only significant difference between both types is the temperature at which they are resistant. Kanthal A1 can withstand temperatures up to 1400°C, Kanthal D can withstand temperatures up to 1300°C [33].

The resistance of the heating element and the voltage across it, determine the heating element temperature. Coil heating temperature is an important operating parameter which affects the aerosol properties i.e. the amount and composition of aerosol emitted from e-cigarettes. Typical resistance values of the first e-cigarette devices are in the range between 1.8 to 2.8 Ohms. However, the resistance of the coil does not only depend on the filament material. A wide variety of coil configurations (Figure 1.3) also possibly contributes to the high performance variability among the different devices, even within the same brands [34]. This shows clearly the importance of product design and its characterization.

Besides the heating element, the power delivered to the heating element equally determines the aerosol production. Batteries of different capacities are available. The batteries of the first generation of e-cigarettes delivered low voltages up to 3.7 V. The later generation devices allow users to adjust the power applied to the heating element to deliver voltages between 3 V and 8 V [22]. The combination of a low resistance heating element and high power supply allow users to produce large amounts of aerosols [35]. However, if the power applied to the coil is too high, this might lead to overheating [36]. One concern related to overheating is, as mentioned before, the dry puffs, which is associated with the release of toxic carbonyl compounds (formaldehyde, acetaldehyde, acrolein) as a result of thermal decomposition of the e-liquid [37].

The latest generation e-cigarette devices have a feature built in to avoid overheating of the coil. The so-called temperature control (TC) allows the user to set up a temperature limit of the coil/aerosol (between 100°C to 315°C) [39]. The power sent to the coil then automatically adjusts to keep the coil at the chosen temperature. The concept of TC is based on the principle that the resistance of certain metals (nickel, titanium, stainless steel) changes significantly and in a repeatable manner when the temperature changes [40]. Because these changes in resistance of the wire are predictable, the power delivery of the battery is adjusted accordingly to maintain the temperature set by the user.

Figure 1.3*: Different coil designs occur in e-cigarette devices. The wire could be flat or round, the space between each winding is variable, the number of winding, etc. The impact of these different coil designs and configurations on the chemical composition of the aerosol is not known yet. Figure adapted from [38].*

E-cigarettes have a broad range of designs. Taken together, it is clear that adequate characterization of e-cigarette design features is necessary to evaluate the potential risks and benefits associated with their use [22].

CHAPTER II - THE REGULATORY CONTEXT OF THE ELECTRONIC CIGARETTE

1 INTRODUCTION

When e-cigarettes were first introduced, their legislative context was not very clear and varied considerably from country to country [41]. In addition, during the course of this project, the regulatory context of the e-cigarette has undergone a radical change, in particular through the adoption of the revised EU Tobacco Products Directive 2014/40/EU (TPD) in 2014 [42]. Here, the TPD and the regulatory situation of e-cigarettes in Belgium before and after implementation of the revised TPD will be discussed.

Regulating e-cigarettes is part of a long history of tobacco legislations. In Figure 2.1, a timeline is given with the major milestones in the EU tobacco legislations from 1983 until 2014. This overview shows that tobacco control measures include more than only tobacco product legislations: i.e. tobacco taxations, advertising and sales promotions, tobacco outlets, smoke-free public spaces, cessation strategies, labelling and packaging, and tobacco industry surveillance.

Tobacco product policies help to limit the health impact of tobacco use by intervening via three major aspects of tobacco products (Figure 2.2) [43]:

(i) Attractiveness: in general, all tobacco products are engineered to make them attractive for example by the addition of flavours and additives. Yet, to discourage tobacco use, the attractiveness of tobacco should be minimized. This can be done by introducing policies to reduce the appeal of products among new users, reduce masking of the tobacco harshness and banning flavourings. Finally by negating the claimed perceptions of safety.

(ii) Addictiveness: nicotine is the primary addictive component in tobacco products. However, on its own, nicotine is extremely harsh and irritating. Tobacco products are designed by manufacturers to ensure high exposure of nicotine. This can be achieved by reducing or masking the harshness and irritation of nicotine and tobacco. On the other hand, high nicotine exposure can also be achieved by increasing the rate and speed by which nicotine is delivered and absorbed. This is done by facilitating the bioavailability of nicotine through manipulation of product chemistry or by addition of compounds which interact with or increase the effects of nicotine. In this case, policy can interfere through a ban of substances that augment nicotine bioavailability and by limiting the nicotine concentration as such.

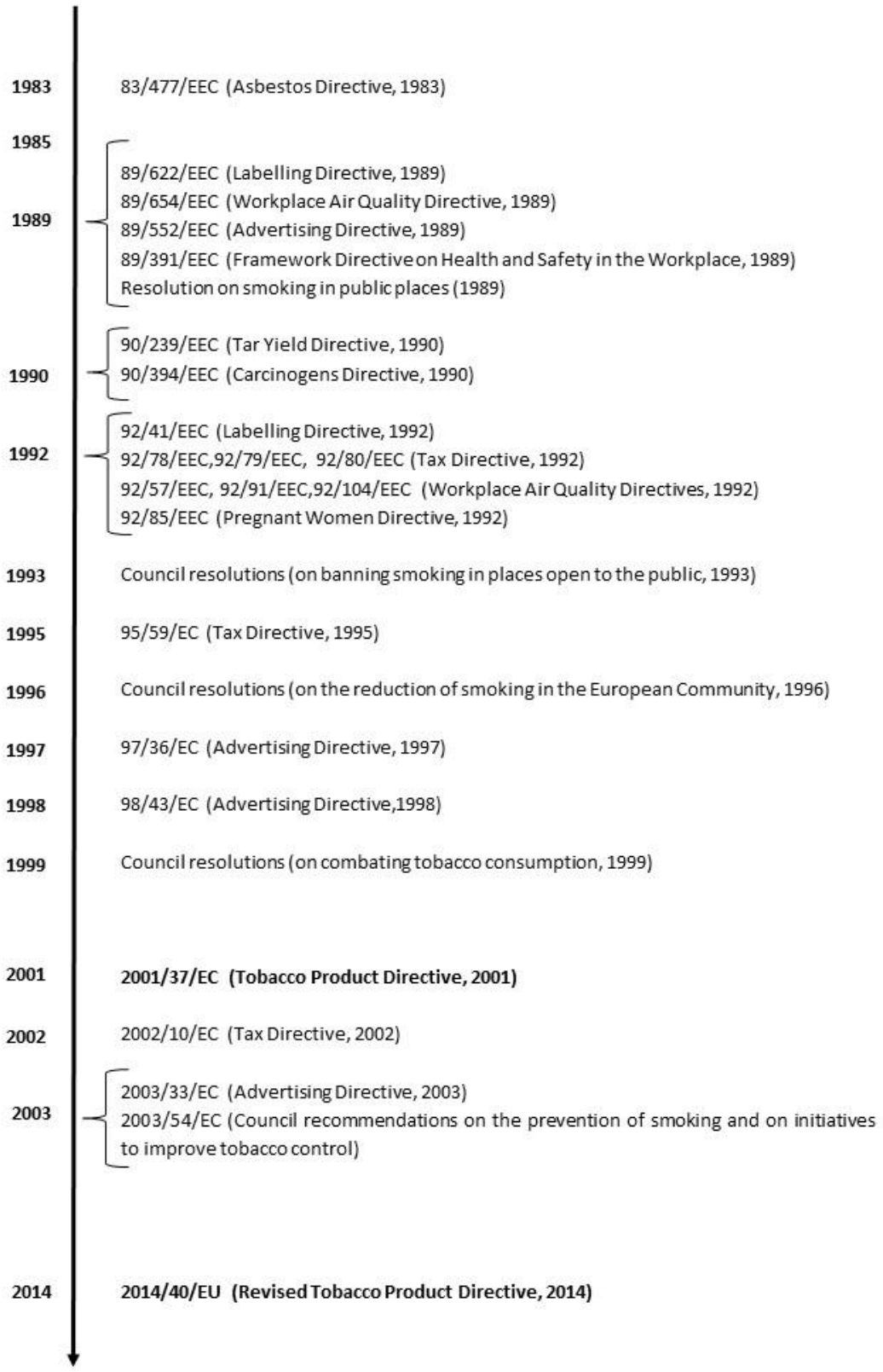


Figure 2.1*: Overview of the major milestones in the EU tobacco legislation from 1983 until the revised TPD in 2014.*

(iii) Toxicity: toxicity of tobacco results from exposure to toxic compounds that are contained in or generated by tobacco products. A way to anticipate for this factor, would be to incorporate policies such as ingredient requirements to reduce the generation of toxic compounds and minimize exposure to toxicants.

2 THE REVISED TOBACCO PRODUCT DIRECTIVE (2014/40/EU)

The revision of the TPD in 2014 was considered as "*the most lobbied dossier in the history of the EU institutions*" [44]. Indeed, Phillip Morris International, the biggest stakeholder of the tobacco industry, employed more than 160 lobbyists and spent around €1.25 million for lobbying on the TPD dossier [45].

The measures taken to regulate tobacco products and e-cigarettes are set out in a Directive instead of a Regulation. A "Regulation" is a binding legislative act which must be applied in its entirety in all the EU Member States. A "Directive" is a legislative act that sets out a goal that all EU countries need to achieve. However, it is up to each EU Member State to formulate their own laws to reach these goals. The choice for a Directive instead of a Regulation also allows Member States to decide on applying the legislation of medicines or to regard the e-cigarette as a consumer product, but imposes certain restrictions with regard to consumer protection and safety.

The following section provides an overview of the most relevant features of the revised TPD for this thesis.

2.1 Definition of an electronic cigarette

In the previous chapter the WHO and ISO definition of an e-cigarette has been given. To even more complicate the topic, it has become clear that "the e-cigarette" as such does not exist and that it covers a wide range of products. Therefore, the revised TPD 2014/40/EU starts with the (only legally binding) definition of an e-cigarette:

"'electronic cigarette' means a product that can be used for consumption of nicotine-containing vapour via a mouthpiece, or any component of that product, including a cartridge, a tank and the device without cartridge or tank. Electronic cigarettes can be disposable or refillable by means of a refill container and a tank, or rechargeable with single use cartridges;"

Although all generations of e-cigarette devices are included, the definition is restricted to nicotine-containing e-cigarettes. Thus, TPD 2014/40/EU is not applicable for non-nicotine containing products,

which means that the regulatory context for these products remains the same as before the revision of the TPD. This may well be a legislative gap and raises several questions concerning e.g. marketing of these products, sale to minors, presence of vitamins and stimulants, …

Furthermore, if an e-cigarette holds a health claim or is intended to be used as drug or medicine, TPD 2014/40/EU is not applicable. The e-cigarette is then subjected to the medicines and medical device legislation. This for instance implies that the e-cigarette may not claim to be a nicotine cessation aid, unlike the classic nicotine replacement therapies:

"The Member States shall ensure that electronic cigarettes and refill containers are only placed on the market if they comply with this Directive and with all other relevant Union legislation. This Directive does not apply to electronic cigarettes and refill containers that are subject to an authorisation requirement under Directive 2001/83/EC or to the requirements set out in Directive 93/42/EEC."

Since the adoption of the revised TPD in 2014, new tobacco products such as the heated tobacco products (HTPs) have meanwhile entered the market [46]. The latter are tobacco leaves that are heated, thus no combustion takes place. Hence, a solid definition for combustion is required. In addition, hybrid tobacco products, using tobacco plant material and nicotine liquid, also became recently available. It is clear that in an updated version of TPD 2014/40/EU, a more general definition is needed to include all novel tobacco products.

2.2 Product notification

TPD 2014/40/EU requires manufacturers and importers of e-cigarettes and refill containers, to submit key information on their products to the competent authorities of the Member States in which they intend to place these products on the market. The purpose of the notification of these products is to assure the quality and safety of these products up to a certain degree [47]. It also allows Member States to monitor the products available on their market. This notification must include information on the ingredients and emissions, toxicological data of the used ingredients, information on nicotine doses and uptake, and a description of the device and production processes. In the Commission Implementing Decision (EU) 2015/2183 of 24 November 2015, a common format for the notification of e-cigarettes and refill containers was introduced [48].

"The competent authorities have the responsibility to monitor notifications and conduct research on the toxicological profile of e-liquids and emissions. Member States should carefully monitor evidence on the health risks of e-cigarette ingredients. As additional evidence emerges, it may be justified for

Member States to prohibit certain ingredients e.g. flavours for use in e-liquids" (as outlined in Recital 47 of the TPD, the responsibility for adopting rules on flavours remains with the Member States).

The notification of the ingredients assures that only permitted ingredients are used. This highly depends on the goodwill of the manufacturers themselves to list all the ingredients used in the refill liquids. Some manufacturers are, however, not eager to do so, because of privacy and confidentiality issues. Therefore, the Commission Implementing Decision (EU) 2015/2183 also states that "*ingredients present at a level below 0.1% in the final product formulation may be deemed confidential or a trade secret. As such, ingredients present at a level below 0.1% in the final formulation are collectively described in the notification by an umbrella term such as e.g. 'strawberry flavouring'*."

2.3 Ingredient requirements

Effective legislation of tobacco product content can reduce the health impact of tobacco use [43]. The three key aspects mentioned in the introduction of this chapter will be used as the guideline for ingredient requirements of tobacco products i.e. attractiveness, addictiveness and toxicity. If products are made less appealing, fewer people will start or continue using tobacco products. If tobacco products are made less addictive, the amount and frequency of use may decrease. If the overall exposure to toxicants is significantly lowered, harm may be reduced.

Unlike the traditional tobacco cigarettes, limitations of ingredients present in e-cigarettes can have an impact on consumer safety. Indeed, the combustion process is the main limitation in traditional tobacco cigarettes. However, in e-cigarettes, the outcome in the aerosol emissions is controlled by the ingredients in the refill liquids. As such, the ingredient requirements/limitations set out in the TPD 2014/40/EU can be divided into four different categories:

(i) Nicotine:

As stated in Article 20(3) nicotine concentrations are limited:

"…Nicotine-containing liquid is only placed on the market in dedicated refill containers not exceeding a volume of 10 ml, in disposable electronic cigarettes or in single use cartridges and that the cartridges or tanks do not exceed a volume of 2 ml…"

"… the nicotine-containing liquid does not contain nicotine in excess of 20 mg/ml;…"

These requirements are included in the TPD 2014/40/EU to mitigate the lethal risks of accidental ingestion of nicotine-containing e-liquids. Nicotine is a known acute lethal molecule (oral LD50 = 0.8–1 mg/kg bw) [49]. The number of cases of lethal nicotine poisoning has increased since the

introduction of the e-liquids [50]. Another justification to avoid excessive levels of nicotine in e-liquid containers is to prevent users to blend their own e-liquid (so-called "Do-It-Yourself" (DIY) e-liquids). Home blending or e-liquid customization not only requires high-concentrations of nicotine, but a number of risks are also associated with DIY e-liquids such as incorrect dilution and nicotine poisoning through dermal exposure [51], [52].

However, due to unavoidable production variability, only a maximum nicotine concentration is given in the TPD, thus without mentioning the acceptable deviation limits, as done in case of pharmaceuticals (i.e. ± 5% for the dosage of the Active Pharmaceutical Ingredient or API). Deviation limits are also not mentioned for the concentration lower than the maximum allowed nicotine concentration. This is important as significant differences between labelled and actual present nicotine concentration is an often occurring problem that will be further discussed in Chapter VII [53]. An important aspect in this matter is the lack of standardized methods to determine the measurement uncertainty. Another factor to be considered is that the clinical impact of different nicotine exposure by inhalation is not assessed and varies from person to person. Thus, the lack of acceptance limits for nicotine deviation has as consequence that nicotine labelling discrepancies have no legal implications.

(ii) Additives:

Article 20(3)(b) of the TPD 2014/40/EU places a requirement on Member States to ensure that the nicotine-containing liquid does not contain additives listed in Article 7(6);

" (a) vitamins or other additives that create the impression that a tobacco product has a health benefit or presents reduced health risks;

(b) caffeine or taurine or other additives and stimulant compounds that are associated with energy and vitality;

(c) additives having colouring properties for emissions;

(d) [for tobacco products for smoking, additives that facilitate inhalation or nicotine uptake]; and

(e) additives that have Carcinogenic, Mutagenic and Reprotoxic (CMR) properties in unburnt form."

The first three additives listed above are all related to reduce the appeal of e-cigarettes. Indeed, the main reason to prohibit vitamins and stimulants in e-liquids is that they might give the perception that these e-cigarette products are healthy or that they may be used for lifestyle purposes instead of nicotine replacement to quit smoking. Consequently, they could also contribute to the normalization of cigarette use, although this has not yet been investigated. The prohibition of colouring additives is to avoid attractiveness towards minors.

(iii) Impurities:

Article 20(3)(d) of the TPD 2014/40/EU states that manufacturers may only use high-purity ingredients:

"…only ingredients of high purity are used in the manufacture of the nicotine-containing liquid. Substances (…) are only present in the nicotine-containing liquid in trace levels, if such traces are technically unavoidable during manufacture;"

This paragraph also provides an exception for impurities that are unavoidable, including some nicotine-related impurities. The Directive is not clear about which impurities are allowed in trace concentrations (nitrosamines, volatile organic components (VOCs), …) and the concentration limits for these impurities in the final e-liquid product. This article further implies that the manufacturer needs to control the production process of the e-liquids and only use high quality raw materials.

(iv) Harmful substances:

The fourth category of ingredients covers all harmful components in its broadest sense (Article 20(3)(e)). Yet, there are no concrete restricted ingredients mainly due to the lack of knowledge:

"… except for nicotine, only ingredients are used in the nicotine-containing liquid that do not pose a risk to human health in heated or unheated form;"

One of the comments on the TPD 2014/40/EU is that it lacks specific guidelines in order to comply to the ingredient requirements such as control of manufacture, enforcement of sanitary conditions, guidance in handling pharmaceutical-grade ingredients and a complete listing of constituents.

2.4 Other public health related requirements

TPD 2014/40/EU also anticipated on the potential risks of oral and dermal e-liquid exposure. Ingestion of nicotine-containing e-liquids can lead to serious poisoning, while dermal contact with e-liquids containing nicotine and other potential irritants can cause skin reactions. In order to mitigate the risk of accidental ingestion or dermal contact of nicotine-containing e-liquids, refill containers and e-cigarette devices should be child-resistant, tamperproof and protected against leakage.

3 LEGISLATION OF E-CIGARETTES IN BELGIUM

In Belgium, the majority of the TPD 2014/40/EU was implemented in the Royal Decree (RD) of 28 October 2016 on the manufacture and marketing of electronic cigarettes or "*KB betreffende het fabriceren en het in de handel brengen van elektronische sigaretten*" [54]. Before the adoption of the

revised TPD, a difference was made between non-nicotine and nicotine-containing e-cigarettes. E-cigarettes containing nicotine were regarded as a medicinal product because they contain an active pharmacological compound i.e. nicotine. Hence, until 2016 the Medicine law of 25 March 1964 was applied for nicotine-containing e-cigarettes. In theory this meant that, after a market authorisation was obtained and once registered as an official medicine, nicotine-containing products were only allowed to be sold in pharmacies. For the registration of medicines, one must provide data to demonstrate the quality, safety and effectiveness of the product. Additionally, e-cigarettes without nicotine but with medicinal claims of smoking cessation or reduction, were as well considered as medicines by their presentation and had to meet the same requirements. Since a license was never requested for any e-cigarette product, an official market authorisation was never granted for nicotine-containing e-cigarettes. So, in practice, this came down to a *de facto* ban of nicotine-containing e-cigarettes. On the other hand, e-cigarettes without nicotine nor with any medicinal claim(s), were only subjected to the obligation to register the chemicals they contain as stipulated in the Registration, Evaluation, Authorisation and Restriction of Chemicals or REACH Regulation (EC) No 1907/2006 of the European Parliament and of the Council of Europe. However, if these non-nicotine containing e-cigarettes were flavoured with tobacco extracts, then they were considered a tobacco product. In that case, the e-cigarettes had to comply with the requirements laid down in the Law of 24 January 1977 on the protection of consumers' health with regard to food and other products, as well as with the Royal Decree of 13 August 1990 on the manufacture and placing on the market of tobacco-based and similar products. In practice, this meant that the legislation of traditional tobacco products was also applied to this type of e-cigarettes, including notification, sales regulation, health warnings on the packaging, prohibition of advertising, etc. Additionally for the second generation e-cigarettes, the electronic part of the e-cigarette was considered a medical device, as defined in the Royal Decree on medical devices of 18 March 1999.

It is clear that the different legislations for e-cigarettes before the adoption of the revised TPD in 2014 were very complicated and ambiguous (Figure 2.3). Hence, the popularity of the e-cigarettes in Belgium was somehow delayed compared to the neighbouring countries such as France, the Netherlands and the UK where e-cigarette use was higher at that time. Because the situation was not the same for the different EU countries, and to continue the commerce of the internal market while ensuring a high level of health protection for European citizens, the original TPD 2001/37/EU was amended with a specific Article for e-cigarette products. Since 2016, the revised TPD 2014/40/EU came into force in Belgium as a Royal Decree (RD). A major modification from the revised TPD that was implemented in Belgium is the sale on distance: internet sale of e-cigarette products is prohibited in Belgium. Additionally, penalties on infringements are specified as well as the fee of notification. In

fact, controversy around the fee of notification was the reason why the first RD of 15 February 2016 was annulled. The first RD thus never entered into force, meaning that in the period between February 2016 and February 2017 e-cigarettes were not regulated and thus not controlled in Belgium, until the renewed RD of 28 October 2016 entered into force and which still applies.

The RD has enforced other requirements compared to the TPD 2014/40/EU that are applicable for e-cigarette manufacturers, importers and vendors in Belgium. The same measures that were applied before the introduction of the revised TPD are still valid. Thus, all chemicals used in the e-liquids need to be compliant with the REACH legislation. This means that registration of the chemicals is mandatory when the produced/imported volume is more than 1 tonnage a year. In addition, e-cigarettes also need to be compliant to the CLP Regulation which is the Classification, Labelling and Packaging of substances and mixtures as described in regulation (EC) No 1272/2008 of the European Parliament and of the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. This means that if the e-liquids contain dangerous substances or mixtures, special labelling needs to be foreseen on the packaging and a notification with the Poison Centre is required. Finally, because e-cigarettes are regarded as tobacco products, it is also prohibited to vape in closed public places, the marketing is limited, discounts are forbidden and sale to minors is prohibited.

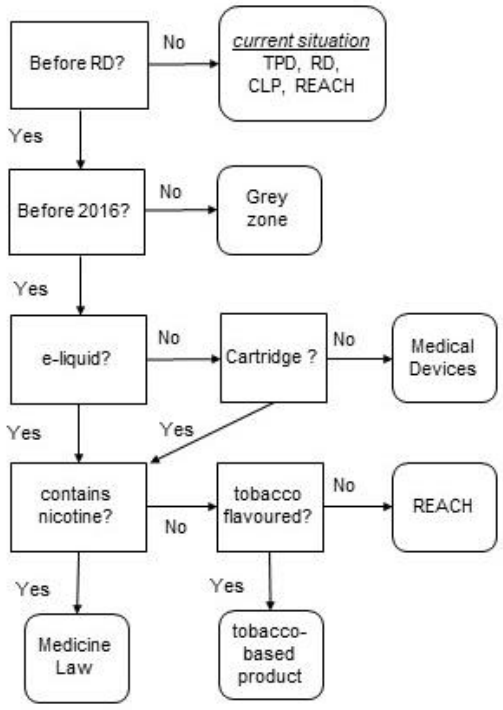


***Figure 2.2**: Overview of the previous and current e-cigarette regulatory context in Belgium (RD = Royal Degree on the Manufacture and Marketing of Electronic Cigarettes, TPD = Revised Tobacco Product Directive, CLP = Classification, Labelling and Packaging regulation, REACH = Registration, Evaluation, Authorisation and Restriction of Chemicals regulation).*

4 CONCLUSION

The regulatory history of the e-cigarette in Belgium illustrates both sides of the debate within the scientific community and public health organisations on what policy strategy to adopt. The nicotine-containing e-cigarettes were first introduced to the Belgian market as a medicinal product. This meant that the precautionary principle was applied as the e-cigarettes were regulated based on absolute safety. Their regulation as a medicinal product also implied an official registration file including full analytical characterization of the product and aerosol emissions, a suit of toxicological tests and proven efficacy and investigated side effects via clinical studies. This allowed health authorities to ensure consumers safety by minimizing the risk of exposure to harmful constituents. The nicotine-containing e-cigarette was also only available in licensed pharmacies, which further reduced as well the risk of "renormalization" or re-acceptance of tobacco use and gateway progression[2] to smoking. However, the opponents of the precautionary principle argue that by making e-cigarettes less accessible and acceptable, it will cause harm as tobacco smokers would not easily have access to a less harmful alternative i.e. the e-cigarette [55]. Thus, the regulatory challenges for the policy makers was to balance between both points of view, which eventually resulted in the TPD. The TPD, however, juggles between harm minimization and the precautionary principle [56]. Therefore, supporters as well as opponents of the e-cigarettes have their own comments on the taken measures. The supporters would like to actively promote e-cigarettes to quit tobacco cigarettes and would therefore classify it differently than the traditional tobacco cigarettes. One of their arguments is that regulating e-cigarettes in a exaggerating way could contribute to the favoured use of conventional cigarettes. The opponents of the e-cigarettes would rather consider e-cigarettes as medicines, as a deficient legislation would renormalize smoking habits and negating the fruits of years of intense anti-tobacco campaigns. It is clear that more research on long-term efficacy and safety is necessary to accomplish an evidence-based health policy and to avoid divergent legislations.

[2] The gateway hypothesis is the theory that the use of e-cigarettes can serve as a gateway to a nicotine addiction and ultimately traditional tobacco consumption.

CHAPTER III – CHEMICAL COMPOSITION OF E-CIGARETTES

Similar to the e-cigarette devices, the e-liquid composition has significantly evolved since the introduction of the e-cigarette. However, manufacturers do not always provide complete information on the chemicals used during their manufacturing process. Therefore in this chapter, the available literature on the chemical composition of e-liquids, common contaminants and compounds of concern that might be present in e-liquids is summarized.

1 MAIN CONSTITUENTS OF E-LIQUIDS

In general, the main ingredients of e-cigarette liquids are nicotine (except for zero-liquids i.e. non-nicotine containing liquids) dissolved in solvent carriers such as propylene glycol and glycerol mixed with concentrated flavourings. The number of chemical substances in the e-liquid can vary widely, depending on the complexity of the used flavouring components. An e-liquid can for example contain up to 60-70 components [57].

1.1 Nicotine

Usually, the main function of e-cigarettes is nicotine delivery to the user. Nicotine (Figure 3.1) is the principal alkaloid component of tobacco, occurring throughout tobacco plants (Nicotiana *tabacum*). Nicotine is known as one of the most addictive substances. The physiological effects of nicotine result from both sympathetic and parasympathetic actions, as nicotine acts as a receptor agonist of most nicotinic acetylcholine receptors (nAChRs) [58].

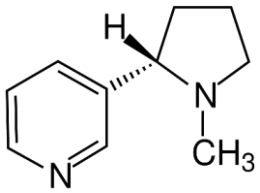


Figure 3.1: *Chemical formula of nicotine.*

Absorption of nicotine can occur easily through the oral cavity and lungs. The absorption across biological membranes depends highly on the pH [59]. Nicotine is hardly absorbed in acidic environments compared to physiological pH (≈ 7.4). Nicotine can also be absorbed through the skin. As such, poisoning after skin contact with nicotine containing pesticides have been reported [60], [61]. There are also case reports of nicotine poisoning in tobacco field workers [62]. Nicotine is metabolized by Cytochrome P450 (CYP450) monooxygenase to cotinine involving a hydroxylation reaction [59]. Furthermore, nicotine is found in several tissues with a high affinity for liver, lungs and brain [63]. Also,

the passage of nicotine through the placenta occurs readily, even in such way that the foetuses are exposed to higher nicotine concentrations than their mothers who smoke [64].

Acute nicotine intoxication occurs mainly through ingestion, but acute systemic toxicity is also possible through inhalation and dermal exposure as mentioned before. Nicotine poisoning results in nausea, abdominal pain, diarrhea, headaches, seating and pallor. More severe poisoning produces dizziness, weakness and confusion which ultimately ends in convulsions, hypotension, coma and death due to depression of the respiratory system [65]. For the determination of the LD50, several acute oral toxicity studies were performed on different species including rat, mouse and dog. However, the available studies are difficult to interpret due to the lack of information on the reported cases and the very large differences between them. Also, the metabolism of nicotine is complex and differs between species meaning there is a high interspecies-variability of the acute toxicity of nicotine. For humans, also several numbers are given for the lethal dose of nicotine in the literature. The often stated value is 1mg/kg bw in adult humans, however, this value is not reliable according to Mayer [66]. Mainly because there are large inter-individual differences in the rate at which nicotine is absorbed and the rate at which it is metabolized and eliminated [67]. Taken into account interspecies and inter-individual differences in sensitivity, the Committee for Risk Assessment (RAC) of the European Chemicals Agency (ECHA) stated that the oral LD50 of nicotine is within a range of 3.34 - 24 mg/kg bw [68], although signs of intoxication could start at 0.3 mg/kg in adults (particularly in people who have not developed an addiction) and 0.2 mg/kg in children [69]. E-liquids containing nicotine can thus cause serious or even fatal poisoning if swallowed by a child and probably also by persons who have not developed tolerance [60].

Evidence of chronic toxicity of pure nicotine (without additive effect of other constituents as for example burnt tobacco) is rather scarce. It is difficult though to investigate the impact of nicotine in tobacco-related diseases, as it is present and absorbed with other potentially harmful substances that occur in tobacco smoke. Nevertheless, studies indicate that nicotine is a teratogen. Indeed, several *in vivo* animal studies show that nicotine has a direct effect on the foetal cardiovascular system and induces structural changes in the foetal lungs resulting in foetal hypoxemia because of the reduced blood flow [70]. There is, however, uncertainty as to whether nicotine is carcinogenic. *In vitro* genotoxicity studies show that nicotine increases chromosome aberrations and sister chromatid exchange frequency in a dose- and time-dependent manner in Chinese hamster ovary (CHO) cells, indicating that nicotine acts as a clastogen [71]. But, findings in this regard have been somewhat inconsistent and according to the IARC there is not enough evidence to consider nicotine as a carcinogen. Yet, other animal studies have shown vascular, liver, pulmonary, renal and neurological

toxicity upon repeated nicotine exposure [72]. Based on currently available literature data, it is difficult to demonstrate the long-term toxicity with the regular use of pure nicotine in humans. Although, there is substantial evidence for effects on the cardiovascular system [73].

Nicotine is intensively investigated in e-liquids. Analytical characterization of nicotine in e-liquids is easy with both gas- and liquid-chromatography. Other non-conventional techniques are used as well such as infra-red, ion mobility and Nuclear Magnetic Resonance (NMR). The chemical analysis of nicotine in e-liquids is further discussed in Chapter IV. Nicotine is mainly quantified in e-liquids to investigate whether the actual concentration corresponds to the claimed concentration on the label and also does not infringe on the maximum limit of 20mg/ml. Previous studies on nicotine analysis show, however, that there are issues with nicotine content variability. In Table 3.1 a summary is given of the nicotine concentration and the deviation from the label as reported in the current literature.

Several studies within different countries have shown that the nicotine content can vary widely among products [74]. Nevertheless, the last few years, we see an evolution towards less discrepancies compared to the period of the first generation e-cigarettes. It is not always easy to verify the accuracy of the nicotine content, especially in e-cigarette cartridges of the first generation. The nicotine level was then reported in a qualitative manner (zero, low, medium, high). Nowadays, the nicotine strength is described quantitatively as mg per cartridge or in mg/ml or mg/g e-liquid. Furthermore, there is also no consensus on what is regarded as a significant discrepancy. Mainly, because the systemic exposure to nicotine not only depends on the actual nicotine concentration of the e-liquid, but also on other factors such as power of the e-cigarette battery, users behaviour and use patterns [75]. Finally, not all of the used methodologies are reported as validated methods. Therefore, lack of standardized analysis methods may also contribute to the uncertainty of the reported results.

With the introduction of the latest generation of e-cigarettes, i.e. the “pods”, also a new way of nicotine use was introduced. Nicotine is generally used in e-cigarettes in its free-base, unprotonated form [92]. Recently, nicotine as a salt with benzoic acid (nicotine benzoate) or levulinic acid (nicotine levulinate) is used in pods. Free nicotine forms a nicotine salt with a weak acid when the device is activated and therefore delivers the nicotine salt in an aerosol form [116]. The nicotine concentration used is higher in these devices compared to e-cigarettes (up to 45 mg/ml). The nicotine plasma concentrations with these devices is also much higher than the nicotine delivery through e-cigarettes [117].

Table 3.1: Literature overview of the nicotine content in e-liquids and accuracy of the nicotine labelling (CA = cartridge, EL = refill liquid/e-liquid, zero-liquids = 0 mg nicotine, DIY = Do-It-Yourself liquids). NA = not applicable, NM = not mentioned

First Author	Year	REF	Analysed samples	Non-conformity zero-liquids (number non-conform/total)	Concentration found	Non-conformity nicotine (>10%) (number non-conform/total)	Deviation	Remarks
Westenberger	2009	[76]	18 CA	4/5	0.01-0.07 µg/cartridge	NA (qualitative label)	NA	
Cobb	2010	[77]	2 CA	NA	NA	NA (qualitative label)	NA	
Trehy	2011	[78]	30 CA 22 EL	5/8 CA 2/5 EL	0.1-21.80 mg/cartridge 12-21 mg/ml EL	22/22 CA 7/17 EL	0.38%-131% CA 54% - 125% EL	
Pellegrino	2012	[79]	2 CA	1/1 CA	NA (qualitative)	NA (qualitative label)	NA	
Goniewicz	2012	[80]	20 CA 15 EL	1/2 CA 1/1 EL	<LOQ - 0.3mg/cartridge	CA 9/18 EL 8/14	-89% - 25% CA 75% - 28% EL	
Cameron	2012	[81]	7 EL	NA	NA	1/2 5 EL qualitative label	79.60%	
Goniewicz	2013	[82]	5 CA	NA	NA	NA (qualitative label)	NA	
Krischner	2013	[83]	6 EL	NA	NA	6/6	50 - 138%	
Kubica	2013	[84]	41 EL	25/41	5.11 µg/g - 339 µg/g	NA	NA	
Etter	2013	[85]	18 EL	NA	NA	1/18	+ 0.5%	
Cheah	2014	[86]	20 CA	1/2	1mg/cartridge	16/18	-89 to 105%	
Visser	2014	[24]	183 EL	3/43	1.2-17.8 mg/ml	67/146	-89.17 to 70%	
Schober	2014	[87]	6 EL	0/3	NA	3/3	16.7%-27.8%	
Hutzler	2014	[88]	28 EL	7/10	0.1-15 µg/ml	qualitative labelled	-	
Hahn	2014	[89]	54 EL	5/23	0.11 - 6.9 mg/ml	no info	no info	in general in agreement with labeling
Farsalinos	2015	[90]	21 EL	NA	NA	9/21	-21% to 22.1%	focus on tobacco flavourings
Geiss	2015	[91]	6 EL	0/2	NA	3/4	13.3% - 14.4%	
Herrington	2015	[57]	4 EL	NA	NA	3/4	17% - 28%	

First Author	Year	REF	Analysed samples	Non-conformity zero-liquids (number non-conform/total)	Concentration found	Non-conformity nicotine (>10%) (number non-conform/total)	Deviation	Remarks
El-Hellani	2015	[92]	17 CA	NA	NA	13/14 NOK 3 qual	-58,9% to -16,3%	
Lisko	2015	[93]	36 (CA and EL)	0/5	NA	16/29	-45% to -13.1%	
Goniewicz	2015	[94]	91 EL	4/28	<LOQ - 0.9mg/ml	25/63	-92,4% to 103,7%	
Kim	2015	[95]	15 EL	0/2	NA	6/13 NOK	-32.2% to 3.3%	mainly measured less than labelled
Davis	2015	[53]	72	0/10	NA	35/56 6 no label	-12.9% to 89.7%	
Kavvalakis	2015	[96]	263 EL	1/2	NM	NM	no details in paper to calculate deviation-	Mean per company of measured concentration reported, no detailed results.
Regueiro	2016	[97]	12 EL	2/4	0.002-12.764 mg/ml	2/8	-40% to -25%	
Crenshaw	2016	[98]	58 EL	4/14	0.001-0.023 mg/ml	19/30	-34% to -11.6%	
Sleiman	2016	[99]	3 EL	NA	NA	3/3	+13 to +78.3%	
Davis	2016	[52]	30 DIY flavourings	4/30	<LOQ (10µg/ml) - 95.4mg/ml	NA	NA	
Famele	2016	[100]	95 EL	6/37	<LOQ - 0.25mg/ml	27/58	-33.3% to -10.7%	
Peace	2016	[101]	27 EL	NA	NA	17/27	-55% to 39%	
Buettner-Schmidt	2016	[102]	93 EL	10/23	<LOQ - 0.48mg/ml	36/70	-66% to +172%	
Medana	2016	[103]	13 EL	3/3	0.011-0.015	6/10	26-79%	
Meruva	2016	[103]	3 EL 3 CA	NA	NA	2/6	-12.50%	application note

First Author	Year	REF	Analysed samples	Non-conformity zero-liquids (number non-conform/total)	Concentration found	Non-conformity nicotine (>10%) (number non-conform/total)	Deviation	Remarks
Ogunwale	2017	[104]	10 EL 2 CA	NA	NA	2/10	17.33% - 25.20%	
Raymond	2017	[105]	70 EL	32/35	<LOQ - 23.91 mg/ml	22/35	-35.31% to +52.37%	
Beauval	2017	[106]	3 EL	NA	NA	0/3	NA	
Dai	2017	[107]	3 EL	NA	NA	1/3	14,2%	
Etter	2017	[108]	34 EL	NA	NA	10/34 NOK	-13% to + 30%	
Omaiye	2017	[109]	125 EL	17/125	0.4 mg/ml - 20.4 mg/ml	NA	NA	
Wenzl	2017	[97]	238 EL	45/91	0.0005 mg/ml - 13.57 mg/ml	66/147	-99.9 to +14.9	
Aszyk	2017	[110]	25 EL	0/4	NA	1/21	15%	
Dai	2018	[111]	16 EL	0/8	NA	3/8	-11.4% to -24.7%	
Abd Rashid	2018	[112]	7 EL	2/7	9.63 - 16.38µg/g	NA	NA	
Bansal	2018	[112]	12 EL	0/2	NA	10/10	12.0% - 17.9%	
Gholap	2018	[112]	10 EL	NA	NA	0/10	NA	
Czoli	2018	[113]	78 CA 88 EL	5/68 NOK	NM	20/73	NM	
Girvalaki	2018	[114]	122 EL	NM	NM	NM	-41.7% to 91.8%	detailed data not given, only means deviations and min-max per EU Member State
Chivers	2019	[115]	10	6/10	0.5mg/ml-2.9 mg/ml	NA	NA	

1.2 E-liquid formulation components

A common misconception within the general population is that the e-cigarette generates nicotine in "water fog". However, e-cigarettes use humectants like propylene glycol and glycerol as solvent carriers to dissolve the nicotine and to produce aerosols that simulate tobacco smoke.

Propylene glycol (Figure 3.2) is a viscous hygroscopic diol widely used in consumer products, foods and medicines. It is odourless and tasteless, making it an ideal solvent carrier for flavours in e-liquids. Moreover, it has excellent solubility properties for many chemicals and produces a visible aerosol when heated. Hence also its use as artificial smoke in theatrical productions. Propylene glycol is mostly used in cosmetics, food and medicines and therefore generally recognized as safe (GRAS). Propylene glycol showed a very low systemic toxicity in animal studies. The lethal dose in humans is estimated to be 15 g/kg bw [118]. However, inhalation of propylene glycol might result in throat irritation and dry mouth [119].

Glycerol (Figure 3.2) is a polyol due to the presence of three OH-groups. It also has hygroscopic properties, but is more oily and thicker than propylene glycol. Like propylene glycol, glycerol is widely used in pharmaceuticals and cosmetics, and in paints, resins and paper. Glycerol is also recognized for low health risks upon human exposure as oral doses of up to 4 g/kg are easily tolerated [120]. The long term effects of both propylene glycol and glycerol via inhalation are unknown [72]. However, at high temperatures both might lead to the formation of highly toxic carbonyl compounds, which is further discussed at the end of this chapter [121]. E-liquid formulations typically contain more propylene glycol than glycerol (30-50%).

Yet another diol has been detected as alternative solvent carrier i.e. 1,3-propanediol (Figure 3.2). It has been suggested that 1,3-propanediol has a better thermal stability and provides higher nicotine delivery compared to propylene glycol and glycerol [122].

Propylene glycol *Glycerol* *1,3-Propanediol*

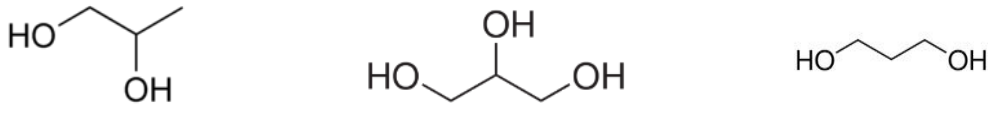


Figure 3.2: *Main solvent carrier components of an e-liquid formulation.*

1.3 Flavours

The main reason for the popularity of e-cigarettes is that it comes in different flavours. In 2007, more than 7000 flavours were available for e-cigarettes [123]. Each flavour in the e-cigarette may be composed of different flavouring components i.e. artificial chemicals, essential oils or herbal extracts [124]. In contrast to e-cigarettes, legislation on flavours in classical tobacco products is stringent and clear: the use of flavourings in tobacco cigarettes is restricted to a certain level which does not contribute to a characterising flavour to the tobacco product itself, as it further encourages the use of tobacco cigarettes and maintains the nicotine addiction of the user [124]. Unlike for e-cigarettes, where flavours as well represent a major part of their attractiveness, and are not restricted by legislation.

Flavourings can be divided in several groups: (i) artificial flavours, (ii) natural flavours and (iii) synthetized-natural flavours. The majority of the used flavourings are food-grade, meaning that they are assessed and permitted for oral use. These flavourings acquired the GRAS status from the US Flavor and Extract Manufacturers Association (FEMA). In Europe, the European Food Safety Authority (EFSA) is responsible for the assessment of food chemicals. Regardless of the approved use in food products, the inhalation safety of these GRAS flavours is not assured because of major differences between both routes of exposure such as the direct exposure to the airways, different absorption and bioavailability and the bypass of the first-pass effect by the liver [125]. Additionally, these flavourings are heated, which might result in toxic thermal degradation products. In this context, a study investigating the production of toxic aldehydes in aerosols suggested a direct correlation with the flavour concentration in e-liquids [126].

The flavouring substances are often not included in the disclosed ingredient list of the manufacturer due to trade secrets. The concentrations of flavourings have been found to vary from 1 to 10% in weight of the e-liquid [127]. The flavouring substances can be divided in 8 chemical groups: aldehydes, ketones, esters, alcohols, acids, terpenes, lactones, heterocycles and sulphur compounds. These flavourings are the major factor that influences the pH of the e-liquid and might therefore also affect the nicotine deposition in the airways and the absorption rates [93].

It is beyond the scope of this chapter to evaluate separately the evidence on the toxicity and safety for inhalation of each flavouring compound, considering the very large number of possible flavourings as well as the fact that not all possible flavouring compounds that are currently used in e-liquids have been identified. In general, flavouring compounds might lead to acute inhalation toxicity [128]. Especially, flavourings from the aldehyde groups are known as irritants of the respiratory tract [129].

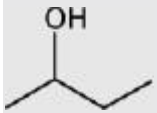
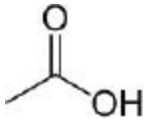
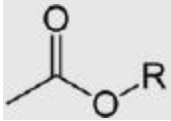
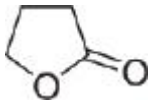
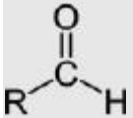
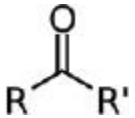
Some flavourings are also known to be substances with sensitizing potency. Examples are given in Table 3.2 which is adapted from the *'Public Health Consequences of E-cigarettes*" report [130]. The most discussed hazardous flavourings in e-liquids are diacetyl and acetylpropionyl. These food additives are used for their characteristic creamy and buttery flavour. Both are mentioned on the GRAS list of FEMA and thus approved for ingestion within certain limits. However, this list is not an indication for safe use in e-liquids. Both diacetyl and acetylpropionyl are possibly associated with lung diseases such as bronchiolitis obliterans [131].

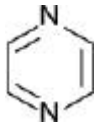
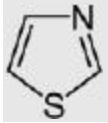
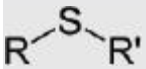
Thus, more research is necessary to exclude acute and local inhalation toxicity of flavourings and to establish their safety upon long-term exposure via inhalation. Additionally, regulatory limits are needed for the most concerned individual flavourings as well as the total (aggregated) content of chemical flavourings.

1.4 Ethanol

Ethanol is considered as one of the constituents of an e-liquid base besides propylene glycol and glycerol. It is identified in the majority of the investigated e-liquid samples [132]. In an acute inhalation study ethanol vapour was found to have a low toxicity [133]. Furthermore, only limited studies are available that investigate the repeat dose toxicity by the inhalation route [134], [135].

Table 3.2: Overview of the most common flavourings per chemical group and indication of their acute inhalation toxicity (LC: lethal concentration). Table adapted from [130].

Chemical Group	Flavouring Chemical	CAS Number	Flavor Type	Respiratory Irritant	Inhalation Toxicity
Alcohols	Geraniol	106-24-1	Floral		
	Menthol	2216-51-5	Mentholic		
	Thymol	89-83-8	Herbal	✓	
	Eugenol	97-53-0	Spicy	✓	
Acids	Butyric acid	107-92-6	Cheesy		Mouse LC > 500 mg/m^3
	Valeric acid	109-52-4	Cheesy		Mouse LC_{50} > 4,100 mg/m^3/2 hours
	2-Methylbutyric acid	116-53-0	Acidic		
Esters	Ethyl butyrate	105-54-4	Fruity		
	2-Methylbutyrate	105-37-3	Fruity		
	Methyl cinnamate	103-26-4	Balsamic		
	Methyl salicylate	119-36-8	Minty		
Lactones	a-nonalactone	104-61-0	Coconut		
	b-decalactone	705-86-2	Coconut		
Aldehydes	Geranial	141-27-5	Citrus		
	Benzaldehyde	100-52-7	Fruity	✓	Mouse LC > 500 mg/m^3 Rat LC > 500 mg/m^3
	Cinnamaldehyde Vanilin	104-55-2 121-33-5	Spicy Vanilla	✓	Mouse LC > 41,700 µg/kg/2 hours Rat LC > 41,700 µg/kg/4 hours
Ketones	Diacetyl	431-03-8	Buttery	✓	
	Acetyl propionyl	600-14-6	Buttery	✓	
	Raspberry ketone	5471-51-2	Fruity		

Chemical Group	Flavouring Chemical	CAS Number	Flavor Type	Respiratory Irritant	Inhalation Toxicity
Oxygen containing	Furfural	98-01-1	Bready	✓	Human TC_{LO} 310 µg/m^3 Rat LC_{50} 175 ppm/6 hours
	5-Methylfurfural	620-02-0	Caramellic	✓	
	Maltol	118-71-8	Caramellic	✓	
Nitrogen containing	2-Acetylpyrazine	22047-25-2	Popcorn	✓	
	2,3,5-Trimethylpyrazine	14667-55-1	Nutty	✓	
	2-Acetylpyrrole	1072-83-9	Musty	✓	
Sulfur containing	2-Isopropyl-4-methylthiazole	15679-13-7	Fruity		
	2-Isobuthylthiazole	18640-74-9	Green	✓	
Mercaptans	Furfuryl mercaptan	98-02-2	Coffee	✓	
	Thiomenthone	38462-22-5	Sulfurous		
	p-Menthene-8-thiol	71159-90-5	Citrus		
Sulfides	Dimethyl sulfide (DMS)	75-18-3	Sulfurous	✓	Rat LC_{50} 40,250 ppm Mouse LC_{50} 3,1620 µg/m^3
	Tropathiane	67715-80-4	Tropical	✓	
	Ethyl vanillin	121-32-4	Vanilla	✓	
	Ethyl maltol	4940-11-8	Caramel		
	Ethyl 3-methyl-3-phenylglycidate	77-83-8	Fruity		

2 IMPURITIES AND CONTAMINANTS IN E-LIQUIDS

Besides the main ingredients listed above, other constituents have also been identified both in e-liquids and their aerosol emissions. The components of concern can be divided in three main categories:

(i) Raw material impurities – these are components related to the main constituents or are present in the raw material of the constituents as contaminants, components from degradation or as by-product from the production process;

(ii) Leachables – metals and other contaminants becoming available by the contact between the liquid and the e-cigarette device;

(iii) Interaction by-products – these are components that are formed from chemical interactions between e-liquid ingredients.

2.1 Raw material impurities

2.1.1 Nicotine-related impurities

2.1.1.1 Minor alkaloids

The majority of the nicotine used in e-liquids is extracted from the tobacco plant and subsequently purified by distillation [136]. Synthetically produced nicotine has only recently become available and is still too expensive to be used in e-liquids or in pharmaceutical preparations [137].

Nicotine is not the only tobacco alkaloid that is present in tobacco leaves. Together with the extraction of nicotine, other minor alkaloids are simultaneously extracted and, depending on the purity of the extract, will still be present in the final nicotine raw material that is used in e-liquids [138]. The most frequently found minor alkaloids are: nicotine-N-oxide, myosmine, beta-nicotyrine, cotinine, nornicotine, anabasine and anatabine (Figure 3.3). These may arise through biosynthetic processes in the plant, by bacterial activity or oxidation during tobacco processing [100]. The European Pharmacopoeia (and United States Pharmacopeia) have set maximum allowed concentration limits for each specified impurity and the total allowed specified impurities, respectively 0.3% and 0.8% relative to the nicotine concentration that pharmaceutical grade nicotine may contain [139]. The analysis of these nicotine-related impurities in e-liquids might be an interesting indicator of the quality and stability of e-liquids as well.

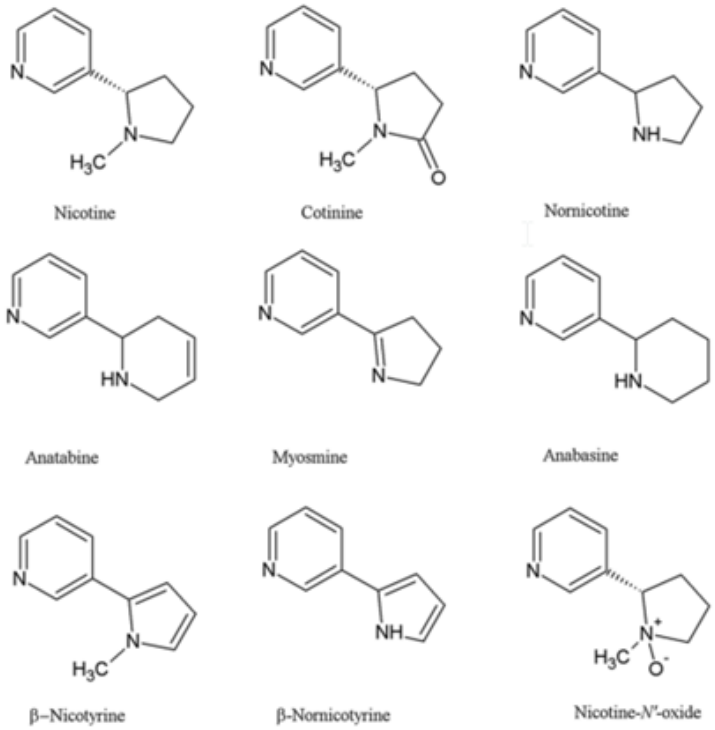


Figure 3.3: *Tobacco alkaloids and potential impurities of nicotine.*

2.1.1.2 Nitrosamines

Nitrosamines are yet other nicotine-related impurities derived from the minor alkaloids. These are potent carcinogenic chemicals formed during tobacco curing, fermentation and ageing [138]. Via nitrosation reactions N-nitrosonornicotine (NNN), N-nitrosoanabasine (NAB) and N'-nitrosoanatabine (NAT) are formed from their corresponding secondary amines (nornicotine, anatabine and anabasine), whereas Nicotine-derived nitrosamine ketone (NNK) is formed from the tertiary amine nicotine (Figure 3.4) [140]. Nitrosamines are mainly associated with tobacco smoke. The low concentrations in e-liquids thus far reported correspond to levels typically found in pharmaceutical nicotine products. Hence, the analysis of nitrosamines appears to be less relevant for e-cigarettes [141].

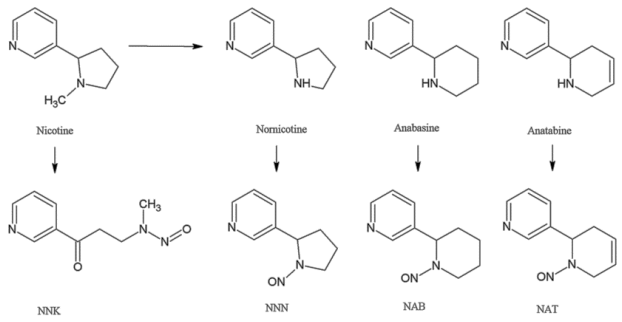


Figure 3.4: *Tobacco nitrosamines and their precursor secondary/tertiary amine. Via nitrosation reactions N-nitrosonornicotine (NNN), N-nitrosoanabasine (NAB), and N'-nitrosoanatabine (NAT) and nicotine-derived nitrosamine ketone (NNK) are formed.*

2.1.2 Diol-related impurities

The solvent carriers propylene glycol and glycerol might also contain contaminants depending on their purity. Indeed, industrial grade of these humectants may contain traces of ethylene glycol (EG) and di-ethylene glycol (DEG) which are known toxicants to humans (Figure 3.5). These are typically impurities that are found by using inferior grade raw materials. Up till now, these impurities have only been reported to be sporadically present in e-liquids [89].

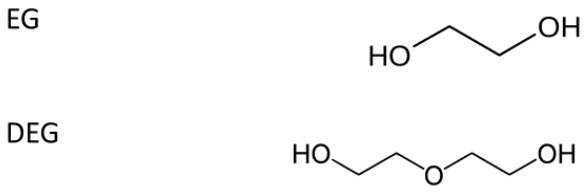


Figure 3.5*: Diol-related impurities. Ethylene glycol (EG) and di-ethylene glycol (DEG).*

Ethylene glycol, which is commonly used as anti-freeze agent, is moderately toxic. When ingested, CNS toxicity (intoxication, lethargy, seizures, and coma), metabolic acidosis, and renal toxicity are reported. Lethal effects in human adults (case reports from accidents, misuse, or suicidal attempts) occur from doses of 1600 mg/kg bw [142]. There are not many studies available investigating the effects of ethylene glycol after inhalation exposure. A study with human volunteers noted irritation symptoms in the respiratory tract [143]. Ethylene glycol has been reported by Hutzler *et al*. as the dominant solvent carrier in five e-liquid products and in lower concentration in other products [88]. Di-ethylene glycol is known for poison epidemics in consumer products as it is highly toxic; ingestion can lead to serious complications that may prove fatal [144]. The toxic dose of di-ethylene glycol has been estimated at 0.14 mg/kg bw and the lethal dose at 1 to 1.63 g/kg bw [145]. The data on toxicity through inhalation exposure is rather scarce [24].

2.1.3 Residual solvents

The major group of impurities related to all the main ingredients are residual solvents present as contaminants from extraction processes. These VOCs are residues from solvents (such as petrogenic hydrocarbons) that are used in the extraction of flavour compounds and nicotine from natural sources [146]. They are especially present in low-quality ingredients (nicotine and flavour extracts).

“Residual solvents” is a term especially used in the pharmaceutical industry for VOCs used during the production process that might be found as residues in the final products. The ICH has created guidelines with a classification and limits for each of these toxic solvents [147]. The guidelines divide the residual solvents into three classes: class 1 consists of solvents that should be avoided in

pharmaceutical preparations due to their high toxicity, class 2 are the solvents that should be limited and class 3 represents solvents with a relatively low toxicity.

The number of reported residual solvents and other VOCs contaminants is limited, because of their instability and volatility in e-liquids under ambient temperature conditions [148]. Nevertheless, VOCs have frequently been reported to be present in the aerosol of e-cigarettes, thus aside their presence in e-liquids. It has been suggested that they are formed by the heating process during vaping of e-liquids [149]. Hence, VOCs are contaminants that can be found in higher concentrations in the e-cigarette vapours compared to the e-liquid itself.

The VOCs which pose the highest health risks are the carcinogens including benzene, toluene, ethylbenzene and xylene (BTEX). An overview of the found concentrations of BTEX in e-liquids is given in Table 3.3. A study on 283 e-liquids showed that these highly toxic VOCs were present in 10% of the sample set, except for benzene [150]. In the same study, 21 concentrated nicotine liquids were also investigated. The results were remarkable: in 20 out of 21 samples toluene, ethylbenzene and xylene were found and in 5 of those samples benzene was present as well. More recently, LeBouf *et al*. investigated VOCs in 146 e-liquids containing different flavours [151]. Benzene was detected in 20 samples, m,p-xylene in 16, o-xylene in 6 and ethylbenzene in 3 samples. The concentrations found were, however, not reported; it was only mentioned that benzene was present in carcinogenic concentrations.

Yet, other VOCs can be present in e-liquids as well. As such, in the same study by LeBouf *et al*., ethanol was the most frequently measured VOC, deliberately added as ingredient to solubilize the flavouring components (see paragraph 1.4).

Isopropyl alcohol was found in 50% of the samples tested, making it the 2nd most common VOC found in e-liquids, followed by acetone. Methylene chloride was also detected in 4 samples, methyl methacrylate in 3 samples, acetonitrile and styrene both in 1 out of 146 samples. In this study, the samples were analysed with headspace-gas chromatography-mass spectrometry (HS-GC-MS). In this case, it is important to correct for the blank background signal as several VOCs such as acetone are also present in ambient air conditions. The concentrations were, however, not reported and it is not known whether the quantification method was validated [151].

Table 3.3: Overview of the publications reporting benzene, toluene, ethylbenzene and xylene (BTEX) in e-liquids. (NA = not applicable)

First author	Year	REF	Number of e-liquids analysed	benzene		toluene		ethylbenzene		xylene	
				frequency %	ppm	frequency %	ppm	frequency %	ppm	frequency %	ppm
Laugesen	2008	[152]	1	100	1.2						
Herrington	2015	[57]	1			1	NA*				
Visser	2015	[24]	60	1.7	9.5	1.7	0.58				
Medana	2016	[103]	16	56.3	0.23 - 29.3	62.5	0.08 - 0.42	87.5	0.08 - 42.3		
Lim	2017	[153]	283			11.3	0.006-0.466	10.2	0.01-.0.092	10.2	0.013-0.159
LeBouf	2018	[151]	146	14	NA*	8.9	NA*	2.1	NA*	11	NA*

* qualitative analysis of the e-liquids

2.2 Leachables

The substances that are inhaled by e-cigarette users are not limited to the e-liquid ingredients and related impurities. The e-liquid also comes in direct contact with the plastic packaging, the e-liquid holder and other parts of the e-cigarette device. Therefore, contamination of the e-liquid is possible with leaching chemicals from the device and packaging. Consequently, these components are not only present in the e-liquid itself, but also in the produced aerosol.

2.2.1 Metals

The coil of the heating element in the e-cigarettes, which is in direct contact with the e-liquid, is the first source of contamination. The contamination occurs before or during the heating of the e-liquid, which can result in metal concentrations in both the liquid in the e-cigarette holder, as well as the aerosol. Recently, higher metal concentrations were found in the aerosol and tank samples compared to the dispenser which demonstrates that coil contact induced e-liquid contamination [151]. The metal concentrations and type of metal elements found in the e-liquids and the aerosol are variable because different alloys are used as coil material. Also metals such as Sn that are present in the joints of the e-cigarette device can leach [154].

The first studies reporting leachable metals focussed on first generation e-cigarettes and their disposable cartomizers that contain the coil and come preloaded with e-liquid [155]. It was found that the concentration of Cr, Ni and Pb exceeded the amount that is typically found in combustible tobacco cigarettes. Only the concentrations of Cd and As were higher in combustible tobacco cigarettes.

Trace elements were also measured in refill e-liquids (which were not yet in contact with the e-cigarette device). Most of the detected elements were found in low concentrations [156]. It concerned the following elements: Al, Cu, Fe, Mn, Ni, Pb, Sn, and Zn. In the aerosols and tank liquid significant higher concentrations were found of heavy metals (Cr, Ni, and Pb) and of metals that may cause toxicity when inhaled (Mn and Zn).

The concentration range of the metals found in the aerosols spanned several orders of magnitude. It should also be noted that the capture method of the metals in aerosols varied in the different studies, demonstrating the need for standardized and validated analytical methods [154], [155], [157]–[159]. However, a biomarker study investigating metal exposure in e-cigarette users and metal biomarkers indicated that e-cigarette users are exposed to high concentrations of Cr and Ni [160]. Both are associated with health risks [161].

Metals are, however, no good quality indicators of the e-liquid itself because their concentration and frequency in the e-liquid is rather low. Nevertheless, they might pose an additional health risk for e-cigarette users.

2.2.2 Phthalates

Other contaminants coming from plastics have also been reported. Diethyl phthalate and diethylhexyl phthalate (DEHP) have been detected in concentration ranges of 0.01–1745.20 mg/L and 0.06–81.89 mg/L in e-liquids [162]. Both are plasticizers used in polyvinyl chloride (PVC) products. They are potential endocrine disrupters and the IARC classified DHEP as possibly carcinogenic to humans [163].

2.3 Interaction by-products

E-liquids are mixtures of different ingredients and contaminants that can be chemically unstable and undergo chemical reactions, thereby forming novel chemical species. One of those interaction by-products has been described by Erythropel *et al*. 2018 [164]. An acetalization reaction can occur between aldehyde flavourings (such as benzaldehyde, cinnamaldehyde, citral, ethylvanillin, and vanillin) and propylene glycol resulting in aldehyde propylene glycol acetal adducts (Figure 3.6). Flavour aldehyde propylene glycol acetals were detected in commercial e-liquids and vaping experiments showed that 50%–80% of the acetals is transferred to the e-cigarette aerosols. It could be shown that these flavour-acetals activated the transient receptor potential TRPA1 and TRPV1 irritant receptors. The TRPA1 and TRPV1 ion channels are identified as the receptors eliciting irritation responses, pain, and cardiovascular reflexes increasing stress and inflammation after exposure to tobacco smoke [164].

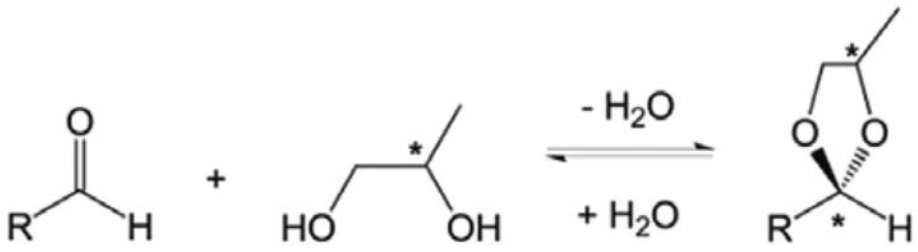


Figure 3.6: *General aldehyde propylene glycol acetal formation reaction involving an aldehyde 1 and PG 2, to form the PG acetal 3. Adapted from [164].*

Taken together, this overview of the possible impurities and contaminants in e-cigarettes is not exhaustive as other impurities are introduced when new products and new ingredients come on the market. Sure is, that if technically possible, impurities should be avoided in the e-liquids. Monitoring

the presence of these impurities is key for the quality of e-liquids, especially for nicotine-alkaloids and residual solvents as their presence points to low quality ingredients.

3 ADDITIVES

Besides the ingredients and impurities, also additives are used in e-cigarettes of which the potential effects on human health when inhaled is not known.

3.1 Stimulants

The first category of additives are stimulants like caffeine and taurine. As mentioned in the previous chapter, these are explicitly forbidden in e-liquids by the TPD because they are associated with energy and vitality and might give the false impression to the e-cigarette users that these are innocent consumer products.

Lisko *et al.* (2016) investigated caffeine in e-liquids with caffeine-associated flavours (coffee, tea, chocolate and energy drink) and e-liquids labelled as energy boosts and caffeinated liquids. They found that 26 of 31 caffeine-associated samples as well as 12 of the 13 energy boost/caffeinated products (label) were positive (3.3 - 347 µg/g and 31 - 9290 µg/g, respectively) [165]. Non-caffeine-associated flavours were not included in the study. The samples were obtained from the US market where, unlike in the EU, the use of caffeine in e-liquids is not prohibited.

Other e-liquid products claim they contain taurine or guarana. There is, however, no information about the transfer of these substances in the aerosols, nor about their bioavailability and safety in humans.

3.2 Drugs

The presence of illicit drugs in e-liquids has been mentioned in several case reports. The most encountered illicit drug used in e-cigarettes is cannabis (oil). There are numerous surveys investigating the prevalence of cannabis vaping, which is the highest in the US and Canada, mainly because of its legalization in some of their states [166]. Compared to cannabis smoking, cannabis vaping results in a reduction of smoke-related toxins and carcinogens and has been proposed as harm minimization strategy for medicinal cannabis users. Studies have shown that vaporized cannabis results in similar pharmacokinetics and plasma concentrations of Δ9-tetrahydrocannabinol (THC) as smoking cannabis [167]. Cannabis has been used in e-cigarettes in different forms such as dry herbs (marijuana head tops), oil concentrates or cannabis-based e-liquids. This is possible with specifically designed and adapted coil heads for e-cigarette devices [168].

Preparations with cannabidiol (CBD) extracts are commercially available. CBD has been claimed to have medicinal benefits such as anti-epileptic, anti-nociceptive and anti-psychotic properties, while ∆9-THC is responsible for the psychotropic effects of cannabis. Analysis of CBD e-liquids has shown that there are significant discrepancies between the labelling of the product and the actual content [169]. The e-liquids contained in 43% of the cases less than 10% of the labelled CBD value. In some cases, even traces of ∆9-THC were detected. Other case reports identified synthetic CBDs such as 5F-MDMB-PINACA (Methyl (S)-2-[1-(5-Fluoropentyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoate) [170] and AB-FUBINACA (N-[(2S)-1-Amino-3-methyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]indazole-3-carboxamide) and ADB-FUBINACA (N-(1-Amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1H-indazole-3-carboxamide) in CBD e-liquids [171]. More recently, opioids were also found in e-liquids. The fentanyl derivate, 4-fluorobutyrylfentanyl, was identified in an e-liquid confiscated as evidence for a fatal intoxication in a concentration of 35mg/ml [172].

In a 2017 US online survey, participants could report for which recreational drugs they have already used electronic vaping devices. These included 3,4-methyl-enedioxy-methamphetamine (MDMA)/'ecstasy' (42.8%), cocaine powder (39.8%), mephedrone (30.9%), crack cocaine (30.5%), synthetic cannabinoid receptor agonists (28.4%), fentanyl (26.7%), heroin (25.8%), alpha-PVP (alpha-pyrrolidinopentiophenone) (25.8%), tryptamines (25.4%), NBOMe (2, 5-dimethoxy-4-bromophenethylamine) (25%), ketamine (24.6%), GHB (gamma hydroxybutyric acid) (0.4%), Magic Mushrooms (0.4%), Solvents (0.4%) and LSD (Lysergic acid diethylamide) (0.4%) [173].

Beginning of October 2019, an e-cigarette controversy was raised in the US. The FDA and the Centers for Disease Control and Prevention (CDC) have both warned for lung injuries and deaths associated with the use of e-cigarette products, more specifically those containing THC [174]. This clearly indicates that the health risks of vaping and inhaling ingredients through e-cigarette devices is not fully unravelled yet.

3.3 Medicines and health supplements

E-cigarette products have been adulterated with active pharmaceutical ingredients as is shown by several case reports. The first report of the identification of medicines in e-cigarettes was by the US FDA in 2010. Both, cartridges and e-liquids, advertised as E-Cialis or E-rimonabant were analysed. Cialis is a commercially available drug containing tadalafil which is used for the treatment of erectile dysfunction. Rimonabant is a drug that was approved for weight loss in Europe, but later withdrawn. E-Cialis did not contain tadalafil (i.e. Cialis™), but the analogue amino-tadalafil. E-cigarette products advertised as containing rimonabant, did contain rimonabant [175].

Recently, as shown in Figure 3.7, a whole new niche of e-cigarettes has emerged that focus on potential health benefits of e-cigarettes with vitamins and health supplements such as vitamin C, vitamin B12, melatonin, etc. (VitaCig, InhaleHealth). These are disposable first generation e-cigarettes which claim to contain several health and herbal supplements. It should be noted that these products are explicitly forbidden in Europe as the revised TPD 2014/40/EU states that the e-cigarette may not contain any medicines, nor vitamins.

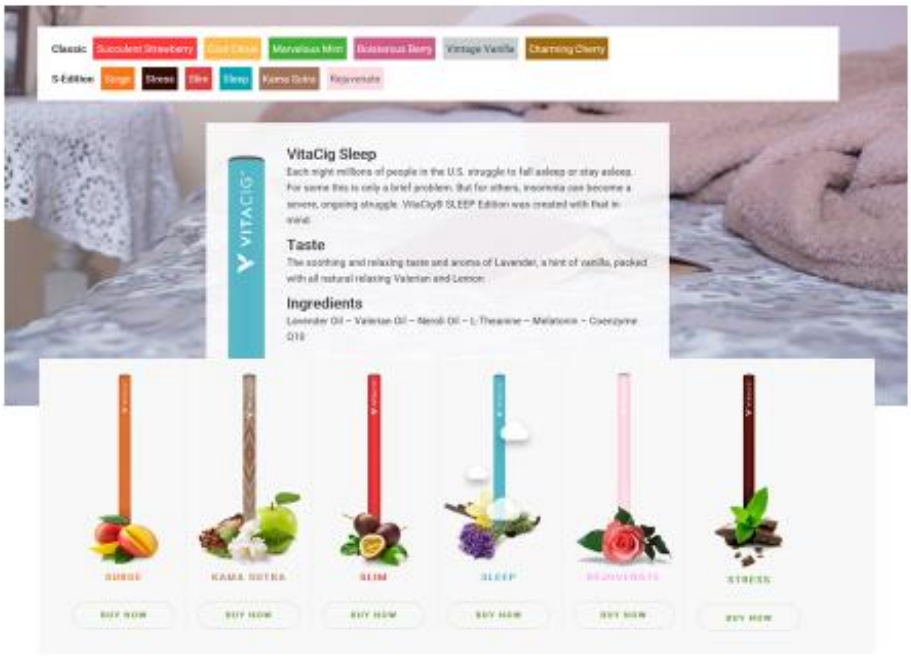


Figure 3.7: *Screenshot from US website (https://www.vitaciggroup.com/) selling vaping products with health supplements.*

4 THERMAL DEGRADATION COMPOUNDS IN E-CIGARETTE AEROSOLS

E-cigarette users are not only exposed to the substances and the contaminants found in the liquids, but also to new by-products formed during the heating of the liquid. The study of thermal degradation products has mainly been focussed on the formation of toxic short chain aldehydes. Only recently other thermal and chemical interaction compounds are being investigated. This research is still in its infancy, though crucial for the health risk assessment of e-cigarettes.

4.1 Short chain aldehydes

Raising attention was a study of the Japanese National Institute for Public Health claiming that e-cigarettes are more dangerous than combustible tobacco cigarettes [176]. This conclusion was based on the significantly higher amount of formaldehyde present in the aerosols of e-cigarettes compared to tobacco cigarettes. Formaldehyde is a Class 1 carcinogen according to the IARC [177] and one of

the three carbonyl compounds formed as thermal by-product from the e-liquid carriers propylene glycol and glycerol. Acetaldehyde and acrolein are also formed according to the scheme in Figure 3.8. The user is aware when inhaling high formaldehyde concentration as this is experienced by the user as an extreme bitter taste in the mouth [27]. These puffs are also called dry puffs as mentioned before.

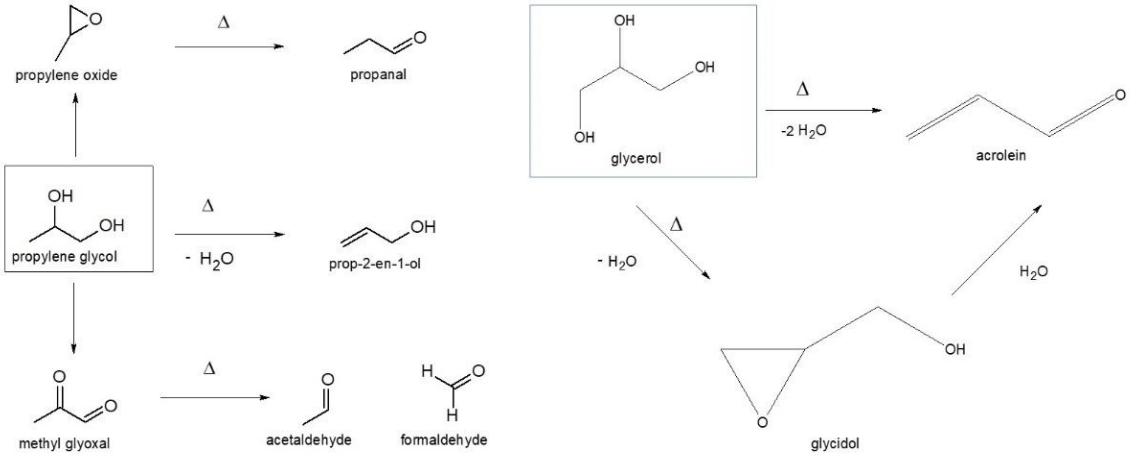


Figure 3.8: *Postulated pathways of by-products formed during thermal dehydration of propylene glycol and glycerol. Figure adapted from [99].*

The aerosol concentrations of these components vary according to several factors, but mainly depend on the temperature of the heating element in the e-cigarette [29]. Also recently, investigators have found that the carbonyl concentration in the aerosol is higher in the presence of flavourings, suggesting that not only the e-liquid carriers contribute to the carbonyl formation, but other ingredients as well [126]. Nevertheless, more investigation is needed to model and predict the carbonyl formation in order to develop e-cigarette models and liquids with minimal carbonyl exposure for the e-cigarette users. Some authors suggest that the carbonyl concentration to which the e-cigarette users are exposed is not significantly higher than the concentration to which humans are exposed in every-daily life from the environment [178]. However, no consensus has been reached yet on the impact of carbonyls from e-cigarette exposure.

4.2 Furans

Thermal degradation of other ingredients than the humectants is also possible during vaping. Soussy *et al*. (2016) investigated the presence of toxic furans such as 5-hydromethylfurfural and furfural in the emissions of e-cigarettes [179]. Both result from the thermal degradation of sugars and are associated with inhalation toxicity and genotoxicity. The presence of these furans is correlated with the power applied on the e-cigarette and the initial concentration of sweeteners in the e-liquids.

5 CONCLUSION

In this chapter, an overview is given of the chemical composition of e-liquids. This composition has significantly changed since the introduction of the e-cigarette and is still evolving (e.g. nicotine salts in pods). Yet, the main ingredients of e-liquids are nicotine (ex. zero-liquids), flavourings, propylene glycol and glycerol. Available studies indicate three main quality and/or safety problems regarding e-liquid ingredients:

i) discrepancies in nicotine labelling of e-liquids;

ii) the use of flavourings that are potential inhalation toxicants with diacetyl and acetylpropionyl as best-known examples;

iii) typical contaminants, impurities and (thermal) degradation products of each main ingredient.

These three issues (nicotine, flavourings and impurities) will provide the basis for our general strategy to assess the quality and safety of e-liquids. This review of e-liquid constituents also shows that HPHC, the most hazardous components in traditional tobacco smoking such as heavy metals and polycyclic aromatic hydrocarbons (PAHs) are not common in e-cigarettes. Indeed, they are not present in the e-liquid as such or at least in much lower concentrations than in tobacco cigarettes.

Very recently, the use of additives such as CBD and THC in e-cigarettes has raised some concern and is being linked to a new lung disease called EVALI which is an acronym for E-cigarette, or Vaping, product use Associated Lung Injury, causing deaths in the US [180]. This underpins that the use of additives in e-cigarettes is not without harm. Inhalation of these additives and their thermal degradation products could be life-threatening.

CHAPTER IV - REVIEW OF ANALYTICAL METHODS USED FOR E-LIQUID CHEMICAL ANALYSIS

The first report on the content of the e-cigarette, was published in 2008 by Health New Zealand Ltd in the context of the safety assessment of Ruyan EC cartridges, i.e. the first patented and launched cartridges [152]. Since the introduction of the e-cigarette, available literature on the chemical analytical characterization of the e-liquids has grown rapidly with each year more publications reporting (novel) analytical methods for the chemical analysis of the composition of e-cigarettes (Figure 4.1).

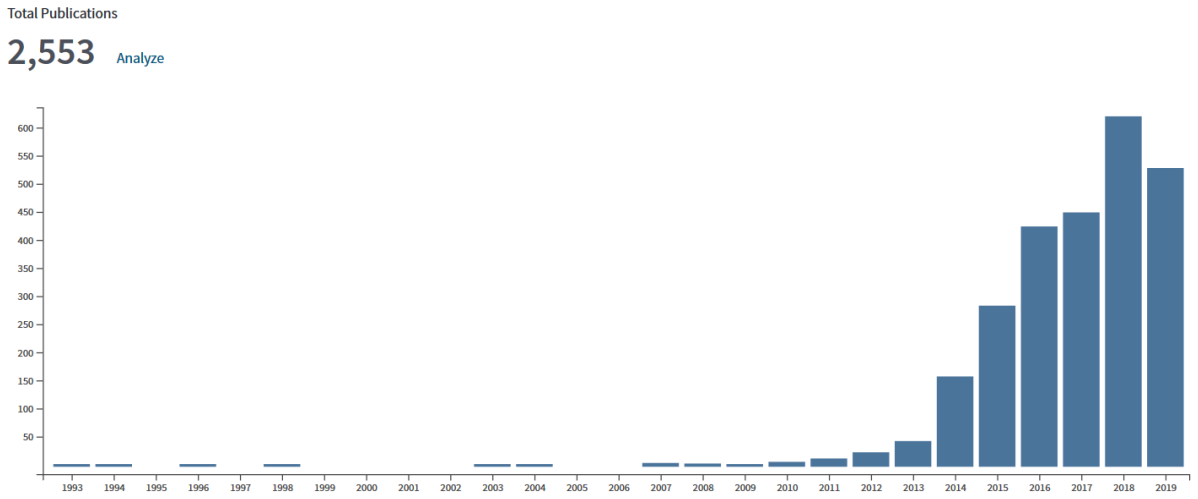


Figure 4.1: *Number of scientific publications per year with as topic e-cigarettes (reported in Oct 2019). The literature review was conducted using the databases Pubmed, Web of Science and Google Scholar with the keywords "electronic cigarettes", "e-cigarettes" and "ENDS". The search was limited to English publications and included publications until 01/08/2019 . Official reports of governmental organizations were also included.*

This chapter reviews methods developed for the analysis of e-liquids (not the aerosols), more specifically components that could be of interest as quality indicators to be able to compare e-liquids as discussed further in this PhD thesis. Thus, analytical methods are only reviewed for the analysis of nicotine and nicotine-related impurities. A section is also included on methods used to analyse flavours, more specifically diacetyl and acetylpropionyl.

1 NICOTINE

Since the introduction of e-cigarettes, the most investigated component in cartridges and e-liquids has been nicotine. Similar to active pharmaceutical ingredients (API) in medicines, nicotine can be regarded as the principal quality indicator of the e-cigarette and hence its presence and dosage need to be guaranteed. The analysis of nicotine has been established in other (more complex) matrices such as tobacco, chewing gums, transdermal patches, biological matrices (hair, blood, urine, etc.) [181],

[182]. Only the methods described for the analysis of nicotine in cartridges and e-liquids are discussed here.

1.1 Qualitative screening of nicotine in e-liquids

With the introduction of the e-cigarettes on the market, qualitative methods for screening and detection of nicotine were particularly developed in the beginning as they were more relevant at that time (Table 4.1). In the period before the implementation of the TPD, nicotine-containing e-liquids were prohibited in several EU countries, also in Belgium. Therefore it was necessary to screen zero-liquids for the presence of nicotine. The presence of nicotine in zero-liquids may originate from different sources [183]. If high doses of nicotine are found in zero-liquids, it might concern intentionally added nicotine wrongly labelled in order to sell nicotine containing e-liquids under the counter, thus illegally. It could also be that the manufacturer has made a labelling error during production. In addition, nicotine may also be present in trace concentrations in the zero-liquids because of contamination during manufacturing or because of the use of natural tobacco extracts as flavourings.

The classical analytical approaches (liquid chromatography (LC), gas chromatography (GC)) are used as screening methods, preferably with MS-detection for identification. In some cases the identification is solely based on the retention times of the target component (FID, NPD, UV). These methods are further described in the section on quantitative methods. They can only be conducted in an analytical laboratory. If these methods are only applied for screening, they could be fast and easy, depending on the sample preparation and whether or not nicotine is the only component of interest. The sensitivity of the methods is widely variable. Not only the screening limit is of importance for the detection of trace concentrations in e-liquids, also the selectivity of the method should be established to assure the correct identification of nicotine. In Table 4.1 an overview is given of methodologies for qualitative detection of nicotine.

Direct Analysis in Real Time MS or DART-MS has been suggested as a fast screening method by Peace *et al*. (2016) using Time-Of-Flight (TOF) as mass analyzer for identification [101]. It is an excellent first tier screening method. However, no screening detection limit was reported, making it difficult to compare its sensitivity to other methods. Another issue concerns the selectivity; the resolving power was only 6000 FWHM (Full width at half maximum) and the components were detected when their mass was within 5 milli mass unit (mmu). Other components with the m/z within 5 mmu of nicotine could be falsely identified as nicotine. Therefore, confirmation is necessary using MS in full spectrum

mode or high-resolution mass spectroscopy (HRMS). Nevertheless, the method allows a fast analysis as it shows the mass spectrum in real time and requires no sample preparation.

In the context of customs control, more easy-to-use analytical methods with minimal sample preparation are necessary in order to conduct a first screening. In some countries the presence of nicotine in e-liquids is forbidden. Therefore, e-liquids ordered online can be intercepted at customs to check their compliance towards labelling and to see whether they are actually nicotine-free. Pilon *et al*. developed a method using ion mobility spectroscopy (IMS) for the screening of nicotine in e-liquids [184]. The method uses a simple sample preparation followed by the analysis of the resulting solution in a commercially available instrument for use in the field. The main limitation of this screening method is that it is not applicable to detect trace concentrations (LOD = 0.05 mg/ml) as it is developed to identify e-liquids containing at least 3 mg nicotine per mL. Another alternative for the detection of nicotine in e-liquids outside the laboratory is with the use of infrared spectroscopy. In 2016, we evaluated the use of attenuated total reflectance-infrared spectroscopy (ATR-IR) and near infrared spectroscopy (NIR), combined with chemometrics for the discrimination between nicotine containing and non-nicotine containing samples [185]. Both ATR-IR and NIR could be used for the discrimination when combined with the appropriate chemometric techniques. When a large enough training set can be established, the interpretation can be fully automated, making the presented approach suitable for on-site screening of e-liquid samples.

Table 4.1: Overview of fast, qualitative screening methods for nicotine. SDL = screening detection limit. NA = not applicable.

Author	Year	REF	Analysis	SDL
Peace *et al.*	2016	[101]	screening DART-MS	not reported
Pilon *et al.*	2016	[184]	IMS	±0.05 mg/ml
Deconinck *et al.*	2016	[185]	ATR-IR and NIR	NA

The above mentioned methods are not sensitive enough to detect trace concentrations of nicotine in e-liquids. Therefore, a follow-up with classical chromatographic methods is still necessary. Nevertheless, easy, fast and hand-held methods to discriminate nicotine and non-nicotine containing samples are still of importance today. In Belgium it is prohibited to import or buy nicotine-containing e-liquids *via* the internet. The ban on long-distance sale implies that postal packages containing e-liquids are checked at customs. Thus, further development of IR and Raman spectroscopy techniques is still useful in the context of fast and out of the laboratory e-cigarette content analysis.

1.2 Quantitative methods for the analysis of nicotine in e-liquids

The amount of nicotine and compliance with the labelled nicotine concentration is an important quality parameter. In this context, several methods have been developed for the quantification of nicotine in e-liquids and cartridges.

1.2.1 Sample preparation

Both GC and LC methods have been widely described for the quantification of nicotine in e-liquids and cartridges (Table 4.2 and Table 4.3). These methods require a sample preparation before injection. The analysis of cartridges usually requires an additional extraction from the cartridge with organic solvent (methanol) to extract the nicotine from the pad, the plug and the cartridge itself. The results are reported as mg/cartridge. The most applied sample preparation for e-liquids is the dilute- and-shoot approach. E-liquid is diluted and subsequently injected. The diluent can vary from mobile phase, methanol (MeOH), acetonitril (ACN):water but also alkaline solutions (ammonia, ammonium acetate) or acidic diluents (ACN:phosphoric acid) are used. Alkaline extraction is used to convert all the nicotine to its free form whilst acidic extraction is used to convert nicotine to its acidic form. Hutzler and Lisko, described a whole liquid-liquid extraction (LLE) with alkaline solution before injection in GC [88], [93]. An alkaline extraction seems less relevant for LC-analysis in case of reversed phase column as nicotine is converted to its free (base) form using a mobile phase with alkaline pH.

E-liquid solutions are not often clear and homogenous, but more likely oily and cloudy, some methods reported an additional filter step [186], probably to avoid particle contamination in the instruments. Alternatively, a guard column is used in some LC-methods to avoid the continuous decrease of column affinity by particle contamination [100].

The nicotine concentration of the samples is expressed as mass of nicotine per volume e-liquid. From a practical point of view, working on a volume-based manner such as pipetting of e-liquids is considered not precise enough due to the viscosity of the e-liquids. Hence, errors are easily made because pipetting of small volumes is not accurate. Goniewicz used reverse pipetting (i.e. the volume aspirated to the tip is bigger than the volume delivered to the receiving vessel) [80]. The excess of volume compensates for the liquid that remains as film inside the tip during dispensing. However, this way of working, is still not as accurate as the weigh-based approach. In order to be able to recalculate the nicotine concentration from a weight-to-weight unit to a weight-to-volume unit, the density needs to be determined.

1.2.2 Gas-chromatography (GC)

Nicotine is volatile and relatively thermally stable, and therefore lends itself easily to be analysed by GC. Several GC-methods have been developed. In Table 4.2 a summary is given.

For maximum selectivity, nicotine is determined by GC-MS. Quantification is performed in Single (or Selected) Ion Monitoring (SIM) mode using external calibration or in some cases in Total Ion Chromatogram (TIC) mode with internal normalization methods, where response factor values determined in advance are used with the peak area. The method to analyse nicotine concentrations in e-liquids developed and validated by Goniewicz [80] has been used by several others [95], [113]. The detection of nicotine is done by a nitrogen–phosphorus detector (NPD) which is also known as thermionic specific detector (TSD). This type of detector is a selective detector for nitrogen and phosphorus-containing compounds, and is therefore more selective for nicotine (and other alkaloids) compared to a flame ionization detector (FID) detector. The latter has been used with e-cigarette cartridges. The identification is mainly based on the comparison of the retention time. Thus, in the presence of other components, the selectivity of the method is not assured.

The GC-FID methods have also been used for the simultaneous analysis of nicotine and the e-liquid solvents propylene glycol and glycerol. In that case, high polar PEG capillary columns are used [107]. In all other cases (also GC-NPD and GC-MS) non-polar i.e. in general 5% phenyl 95% dimethylpolysiloxane GC-columns are often chosen as these are mainly applied for semi-volatiles and alkaloids. When this type of column is combined with direct injection of the e-liquid matrix, faster deterioration might occur after repeated injections because of propylene glycol and glycerol deactivating the interaction with the active groups on the column [187]. The injection mode of the GC-methods for nicotine e-liquids analysis is a direct split/splitless injection with minimal sample preparation. Therefore, it is questionable whether these methods stay robust and reliable after several sample injections. Hutzler *et al*. used a programmable temperature vaporizing (PTV) inlet for injection, with a temperature program of 45°C increasing up to 320°C by a rate of 12°C/min and held for 5 min, thus not with the intention to eliminate the matrix in the GC-column [88].

The sample preparation for GC-analysis is, as mentioned before, based on a dilute-and-shoot approach. For FID-methods, the solvent used is isopropanol. For GC-MS methods, methanol or dichloromethane are used. To avoid interference, methanol is not recommended when other VOCs such as ethanol are analysed.

Table 4.2: Overview of GC-methods for the quantification of nicotine in e-liquids. (LLE = liquid-liquid extraction, RSD = residual standard deviation, RF = response factor)

Author	Year	Reference	Other target components w/ same method	Analysis	Sample preparation	Runtime (min)	Quantification	Range (mg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Validation
Cobb *et al.*	2010	[77]		GC							
Pellegrino *et al.*	2012	[79]	solvents, flavours	GC-MS		32.5	internal normalization method: TIC signal x response factor				
Cheah *et al.*	2014	[86]		GC							
Visser *et al.*	2014	[24]	glycerol, propylene glycol, (DEG, TEG)	GC-FID						10	
Schober *et al.*	2014	[87]		GC-FID	isopropanol	33.5	internal standard		0.10%		
Hutzler *et al.*	2014	[88]		GC-MS	LLE 1:8 dichloromethane	20.8	SIM internal standard	0.0001-0.005			
Farsalinos *et al.*	2015	[146]		GC-FID	1:50 isopropanol	12	internal standard	0.1 - 2		100	
Geiss *et al.*	2015	[91]	glycerol, propyleneglycol	GC-FID	17x and 667x dilution in isopropanol	21	external calibration	0.4 - 2	1	400	
Herrington *et al.*	2015	[57]		GC-FID	100x dichloromethane	<5	external calibration internal standard	0.016 - 1		16	
El-Hellani *et al.*	2015	[92]		GC-MS	LLE toluene	11	SIM internal standard	0.05 - 1		50	RSD <4%

Author	Year	Reference	Other target components w/ same method	Analysis	Sample preparation	Runtime (min)	Quantification	Range (mg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Validation
Pagano *et al.*	2015	[188]		GC-MS	100x methanol sonicated 20min	11	SIM internal standard	0.01 - 1	0.2	0.6	RSD 5%
Lisko *et al.*	2015	[93]	nicotine alkaloids	GC-MS/MS	28x methyltertbutylether	2.3	MRM	0.05 - 42	50		precision: 3.1-3.4% RSD recovery: 93.7-97.9%
Goniewicz *et al.*	2015	[189]		GC-NPD/TSD	100x methanol	12	internal standard	0.01 - 40		50	RSD% 15-17 and bias% 2%
Crenshaw *et al.*	2016	[98]	glycerol, propylene glycol and water	GC-MS	400x ACN	40	TIC internal standard	0.00125 - 0.100	0.15 ng/ml	0.45 ng/ml	
Sleiman *et al.*	2016	[99]	VOC	GC-MS	40x ACN	17	EIC with external calibration of direct liquid injection				
Beauval *et al.*	2017	[106]		GC-FID	4x isopropanol		internal standard	0.5 - 40		500	RSD 0.7%, acc = <5%
Etter *et al.*	2017	[85]	solvents, DEG, EG, anabasine	GC-MS			SIM		10	100	uncertainty 20%
Abd Rashid *et al.*	2018	[112]		GC-MS	100x dichloromethane sonicated for 20 min	11min	no internal standard RF value and peak area	0.005-0.100	3.48	11.6	

Author	Year	Reference	Other target components w/ same method	Analysis	Sample preparation	Runtime (min)	Quantification	Range (mg/ml)	LOD (µg/ml)	LOQ (µg/ml)	Validation
Chivers *et al.*	2019	[115]		GC-MS	derivatisation with BSTFA dilution not mentioned	70min					
Dai *et al.*	2017 2018	[107], [111]	glycerol, propyleneglycol	GC-FID	100x methanol	8 min	internal standard		40		repeat <2%

The dilutions vary from a factor 4 to 400, with 100 most commonly used. Dilutions are necessary because of the viscosity of the propylene glycol/glycerol matrix, which can result for example in the formation of air bubbles in auto-sampler syringes and may therefore negatively impact the precision of the method. The drawback of higher dilutions is less sensitivity.

The LOQ of the methods (if mentioned) depends on the detector. GC-FID methods have a LOQ for nicotine between 0.01 and 0.5 mg/ml while MS methods are more sensitive (LOQ 0.01-0.1 mg/ml). The GC-NPD method has a LOQ of 0.05 mg/ml. These are all expressed as the instrumental LOQ (without taking dilutions into account). The calibration range of the GC-methods is limited making that high nicotine-containing samples need higher dilutions.

The methods are often either not properly validated or validation data is not mentioned. The validated GC-methods have a RSD lower than 5% for the (intermediate) precision, except for the GC-NPD method of Goniewizc which was within the range of 20%. Recoveries and bias are within 95% and 5%, respectively [190], [191]. In general, the validated GC-methods have an acceptable accuracy and precision. However, it should be emphasized that the majority of the methods have not been validated.

1.2.3 Liquid-chromatography

LC is also useful for nicotine analysis in e-liquids, especially in the case of simultaneous analysis with nicotine-alkaloids. The analytical methods described in this paragraph are methods for exclusive analysis of nicotine (Table 4.3). The first LC-methods developed for the analysis of nicotine in e-cigarettes were, however, published without methodological details [76], [81].

Nicotine is easy to detect by UV because of its chromophore. The LC-UV (DAD) methods, described for the analysis of nicotine only, are based on the method of Trehy *et al*. for nicotine-alkaloids analysis [78]. Davis *et al*. combined the HPLC-DAD quantification with a preceding confirmation using GC-MS to assure the identification of nicotine [53]. The main disadvantage of DAD-detection is the low selectivity of the method and interference. The resolution and peak purity of the nicotine peak need to be assured as interference with other nicotine-impurities and flavour components is possible.

Because of this selectivity issue with DAD-detection, Gholap *et al*. developed an HPLC-DAD method assuring a high peak purity of the nicotine peak [192]. They first investigated the separation of nicotine on a C18-column, as this works for most methods. Nicotine was eluted as a single peak, however, the peak purity criteria were not met, indicating that another component was eluting at the same time. Acceptable peak purity for nicotine was achieved using a phenyl column, resulting in a better

separation of the flavours from the nicotine peak. Additionally, the robustness of the method was evaluated for ± 2% changes in organic mobile phase ratio to modify the organic polarity in order to achieve better resolution between nicotine and co-eluting flavours. Another recommended factor which should be included in the evaluation of the robustness of the method, is the pH of the mobile phase as it has a significant impact on the retention time. The pH of the mobile phase, used for nicotine analysis with DAD-detection and a C18-column, is between pH 7.6 to 10. In case of MS-detection, resolution is less of an issue, thus the mobile phase is chosen in function of the ionization. The use of MS-detection assures optimal selectivity, in both SIM or MRM mode. Kavvalkis *et al*. validated a method for the quantification of nicotine in SIM mode with a LOQ of 0.07 µg/ml [96].

The sensitivity of the LC-DAD was variable with a LOQ of 10 µg/ml to 0.45 µg/ml. For quantification of nicotine in commercial e-liquids, the sensitivity of the methods is acceptable as the minimal nicotine concentration found is often 3 mg/ml (1:100 dilution is still 3 times higher the LOQ of the method). When trace concentrations need to be quantified in zero-liquids, more sensitive methods are required. Kubica *et al*. developed and validated therefore two LC-MS/MS methods with LOQ of 0.013 µg/ml in MRM mode [84]. The accuracy at LOQ level and matrix effects were not investigated, even though these are common issues using MRM for quantification. The use of an internal standard (IS) is often needed in case of LC-MS(/MS) analysis because of matrix effects. Otherwise, high dilutions are recommended to eliminate the matrix effect, which results in loss of sensitivity. Most efficient is the use of an isotope-labelled IS such as d4-nicotine [101]. The use of a less specific IS is also possible which is also less expensive [84], [96].

For the analysis of nicotine alone, short methods are applied as there is only one peak of interest. The used LC-UV methods were isocratic with a runtime between 8 – 13 min. The LC-MS/MS methods were faster with runs between 2 – 4 min.

As mentioned before, the majority of the described methods did not include validation data. The LC-UV methods of Bansal *et al*. and Gholap *et al*. were properly validated with an accuracy and RSD% for precision below 2% [192], [193], while the MS-methods of Kubica *et al*. and Kavvalikis *et al*. obtained a RSD of below 2% and 3.6%, respectively [84], [96]. Thus, the LC-methods for nicotine analysis are more accurate and precise compared to the GC-methods. (U)HPLC-DAD is the most efficient method for the quantification of nicotine in e-liquid samples (robust, fast, cheaper than MS, less matrix effects), if the interference of nicotine-related components and flavourings are kept to a minimum (i.e. high peak purity and resolution).

Table 4.3: Overview of LC-methods for the quantification of nicotine in e-liquids. (RSD = residual standard deviation, SPE = solid phase extraction, arb = arbitrary, ACN = acetonitrile, MeOH = methanol)

Author	Year	Reference	Analysis	LOD/LOQ	Range	Runtime	Sample preparation	Details/ remarks
Westenberger *et al.*	2009	[194]	HPLC-UV	NA			10%ACN/ 1% phosphoric acid extraction and methanol extraction (higher recovery with methanol extraction)	no details mentioned
Cameron *et al.*	2012	[81]	LC-MS/MS (ESI)	NA			not mentioned	the replicates of the sample A had an RSD of 13%
Kirschner *et al.*	2013	[83]	LC-TOF-MS (ESI+/-)	NA			dilution-and-shoot (methanol) + isotope dilution	
Kubica *et al.*	2013	[84]	RPLC-MS/MS HILIC-MS/MS (ESI)	LOD 4 ng/ml LOQ 13 ng/ml			dilute-and-shoot	precision: RSD <2%
Davis *et al.*	2015	[53]	HPLC-DAD	LOQ 10 µg/ml / LOD 0,05 µg/ml	0.1 – 1 mg/mg		dilute-and-shoot	
Kavvalakis *et al.*	2015	[96]	LC-MS (APCI) SIM mode	LOQ 0.07 µg/ml	0-10 µg/ml	13 min	dilute-and-shoot	precision RSD 3.6%
Davis *et al.*	2016	[52]	HPLC-DAD			32.75 min		
Peace *et al.*	2016	[101]	HPLC-MS/MS	LOQ 0.01 µg/ml (arb.)	0.01 µg/ml – 1 µg/ml	2 min	dilute-and-shoot (mobile phase)	
Buettner-Schmidt *et al.*	2016	[102]	HPLC-UV	not mentioned			SPE for cartridges	
Raymond *et al.*	2017	[195]	HPLC	LOD 0.01 mg/ml LOD 0.05 mg/ml			dilute (MeOH)	

Author	Year	Reference	Analysis	LOD/LOQ	Range	Runtime	Sample preparation	Details/ remarks
Omaiye *et al.*	2017	[109]	HPLC-DAD	LOD 0.05 µg/ml LOQ 10 µg/ml				
Bansal *et al.*	2018	[193]	HPLC-DAD	LOD 0.07 µg/ml LOQ 0.3 µg/ml	0.78 - 50 µg/ml	8 min	zero-liquids undiluted/ dilute-and-shoot	Accuracy <1% Precision RSD <2%
Gholap *et al.*	2018	[192]	HPLC-DAD	LOD 0.07 µg/ml LOQ 0.45 µg/ml	0.4-500µg/ml	12 min	dilute (100x) and shoot	accuracy <2% precision RSD 2%
Girvalaki *et al.*	2018	[114]	LC-MS					Precision RSD 3.6%

1.2.4 Alternative methods

Next to GC and LC methods, other methods have been developed for the detection of nicotine in e-liquids and cartridges. Both, Crenshaw *et al*. and Hahn *et al.*, developed a NMR method for the detection of nicotine in e-liquids [89], [98]. The advantages of NMR are simple sample preparations, short analysis time (<1 min) and simultaneous detection of other components. However, the LOD is high compared to other methods, meaning that the method is less relevant for the detection of trace amounts.

2 NICOTINE-RELATED IMPURITIES

The investigation of nicotine-related impurities is necessary to assess the quality of the used nicotine and the stability of the nicotine in the e-liquid matrix. Also, some nicotine-related impurities including nicotine-alkaloids are important to investigate because of their potential toxicity.

Nicotine alkaloids were first investigated in e-cigarettes by the FDA in 2009 [194]. Westenberger *et al*. used a GC-MS/MS method for screening of alkaloids in e-cigarette cartridges. The report did not include a quantification of the impurities. A GC-method was also developed by Lisko *et al*. for the quantification of nornicotine, myosmine, anabasine, anatabine and isonicoteine and was performed in MRM mode [93]. The method was adapted from a methodology that was initially developed for the analysis of tobacco plants. Both methods were not specifically validated for e-liquids, which is a reoccurring issue with methods for the analytical characterization of e-cigarettes [196]. While the analysis with GC is feasible for nicotine due to its volatility [188], simultaneous analysis with its related alkaloids is, however, not possible because of the thermally unstable alkaloid nicotine-N-oxide [197]. Other disadvantages of GC-tandem MS methodologies are the insurmountable use of IS, in this case isotopically labelled nicotine and nornicotine.

LC is a more suitable alternative for the simultaneous analysis of nicotine and its related substances. The most easy, robust and straightforward method is LC with UV detection. The quantification of nicotine is easily done by UV detection because of its chromophore. Both Etter *et al*. and Trehy *et al*. have developed an (U)HPLC-DAD method. Trehy *et al*. adapted the USP method and Etter *et al*. the Ph.Eur. method for nicotine-related substances. Etter *et al*. included other nicotine-alkaloids as well such as both isomers of nicotine-N-oxide, norcotinine, β-nornicotyrine and 1-methyl-3-nicotinoylpyrrolidine (MNP). While Trehy *et al*. only included cotinine, anabasine, anatabine, myosmine and β-nicotyrine. The sample preparation is similar to the nicotine analysis: dilute-and-shoot with no use of an IS. Etter *et al*. were not able to provide a method validation, though the LOD was determined to be between 0.01 – 0.03 µg/ml. Trehy *et al*. developed two methods, one with an

isocratic elution and one with gradient elution profile. The recovery of the gradient elution method was within 2% and the RSD of repeatability was less than 0.5% for the 6 components. During method development, it was found that the pH of the eluent greatly impacted the resolution, which is of importance for UV-detection methods. In this case, robustness testing would be recommended to assure that the capacity of the method remains unaffected by small (but deliberate) variations in method parameters. The main disadvantage of DAD-detection methods is the selectivity. There are different pyridine-type alkaloids with the same characteristic UV-absorption spectrum, besides the presence of other flavours with chromophores which can potentially co-elute with the target components, underpinning the fact that peak separation and resolution are important for LC-UV methods.

The most prominent technique used so far is targeted LC-MS/MS in multiple reaction monitoring (MRM) mode, which displays a much higher specificity than through UV-detection [103], [197]–[201]. The presence of other flavours can easily be circumvented with the targeted approach. LC-MS/MS, however, is more susceptible to matrix effects due to interference with propylene glycol and glycerol. The LC-MS/MS methods used for the quantification of nicotine alkaloids are also typically dilute-and-shoot methods. To eliminate the matrix effect of propylene glycol and glycerol, high sample dilutions and/or expensive deuterated IS are used. A summary of the methods used for simultaneous analysis of nicotine and nicotine-alkaloids is given in Table 4.4.

In the next paragraph, LC-MS/MS methods are compared to LC-DAD methods for the analysis of nicotine-related substances in e-liquids. Obviously, runtimes of UHPLC-MS/MS were shorter compared to HPLC-DAD methods. Moreover, achieving a good resolution with DAD-detection is necessary, in contrast to the tandem MS technique in MRM mode that allows quantification even with co-eluting peaks because of the targeted approach. However, a side note needs to be made concerning the selectivity of the MRM approach. Both nicotine and anabasine are isobars, thus both have a similar precursor ion (m/z 163) and elute one after the other. In this case, the resolution needs to be high enough to guarantee separation and different fragmentation ions need to be selected. This is necessary since nicotine is present in much higher amounts than anabasine, which gives a higher risk of co-elution of the large nicotine peak with anabasine and of nicotine carry-over, resulting in potential overestimation of the anabasine concentration.

Table 4.4: Summary of the methods published for the simultaneous quantification of nicotine and minor alkaloids in e-liquids. (NIC = nicotine, COT = cotinine, ANAB = anabasine, β-NIC = β-nicotyrine, ANAT = anatabine, NOR = nornicotine, NNO = nicotine-N-oxide, MYO = myosine, NA = not applicable)

Author	Year	REF	Method	Runtime	Quantification / sample preparation	Extra analytes included ?	Sensitivity	
							Analyte	LOQ
Liu *et al.*	2017	[186]	UHPLC-ESI-MS/MS	18 min	Dilute (1:100)-and-shoot (with additional filter step) The use of isotopically labelled internal standards for each target analyte appears to effectively account for matrix effects.	NA	NIC	2.530 µg/g
							COT	0.781 µg/g
							ANAB	2.390 µg/g
							β-NIC	1.590 µg/g
							ANAT	1.980 µg/g
							NOR	1.990 µg/g
							NNO	5.480 µg/g
							MYO	3.710 µg/g
Regueiro *et al.*	2016	[97]	ESI-DMS-MS/MS (differential ion mobility)	< 1min	Dilute (1:10.000)-and-shoot (with additional filter step)	4,4'-Dipyridyl (DIPY), N-Nitrosonornicotine (NNN), N-Nitrosoanatabine (NATB), N-Nitrosoanabasine (NABS), Cytisine (CYS)	MYO	9.17 ng/mL
							NOR	8.24 ng/mL
							ANAT	7.64 ng/mL
							ANAB	8.25 ng/mL
							NIC	16.5 ng/mL
							COT	8.25 ng/mL
Medana *et al.*	2016	[103]	UHPLC-MS/MS	16 min	Dilute-and-shoot Nicotine-D4 used as internal standard for both nicotine as impurity quantification MRM	N-nitrosonornicotine (NNN)	COT	0.010 µg/ml
							NOR	0.050 µg/ml
							MYO	0.020 µg/ml
							NIC	0.010 µg/ml
							ANAB	0.020 µg/ml

Author	Year	REF	Method	Runtime	Quantification / sample preparation	Extra analytes included ?	Sensitivity	
							Analyte	LOQ
Flora *et al.*	2016	[198]	UHPLC-MS/MS	8 min	Dilute (1 :10) -and-shoot Stable isotope-labeled internal standards for each target analyte MRM except for myosmine, β-nicotyrine, and anabasin	Nicotine, nicotine-N-oxide, cotinine, nornicotine, anatabine, myosmine, anabasine, β-nicotyrine	MYO	0.28 µg/g
							NOR	0.11 µg/g
							COT	0.055 µg/g
							ANAB	0.28 µg/g
							NNO	0.055 µg/g
							ANAT	0.055 µg/g
							β-NIC	0.28 µg/g
Famele *et al.*	2016	[100]	HPLC-MS/MS	12 min.	Dilute (1:1000-1:10.000)-and-shoot For quantification matrix matched calibration curve was used and isotopically labelled internal standard (±)-NC-d4 and dilutions were necessary.		NIC	1.0 ng/mL
							MYO	3.2 ng/mL
							COT	3.3 ng/mL
							NNO	3.1 ng/mL
							NOR	3.1 ng/mL
							BNIC	3.0 ng/mL
							ANAT	6.1 ng/mL
							ANAB	31.8 ng/mL
Meruva *et al.*	2016	[200]	UHPLC-UV-MS (single quad)	7 min	Dilute (1:100)-and-shoot quinoline used as internal standard for both nicotine as impurity quantification		No validation data mentioned 0.5 µg/ml (lowest concentration level)	

Author	Year	REF	Method	Runtime	Quantification / sample preparation	Extra analytes included ?	Sensitivity	
							Analyte	LOQ
Lisko *et al.*	2015	[93]	Two separate methods GC-MS/MS	2.3 min (nicotine) 8.3 min (alkaloids)	Isotopically labeled internal standard, D3-nicotine and D4 – nornicotine Dilute-and-shoot Two separate methods for nicotine and the minor alkaloids. Not all specified minor alkaloids quantified.	isonicoteïne	Nicotine LOD 0.05 mg/g Data validation of minor alkaloids in e-liquids not mentioned (method based on tobacco analysis)	
Trehy *et al.*	2011	[78]	HPLC-DAD	30 min	Dilute-and-shoot Dilution with mobile phase or 10% ACN			(isocratic) µg/ml / (gradient) µg/ml
							COT	1.70 / 0.70
							ANAB	18.5 / 1.30
							ANAT	3.40 / 0.30
							MYO	3.30 / 0.30
							NIC	104 / 0.25
							β-NIC	0.96 / 0.40
Etter *et al.*	2012	[85]	UHPLC-DAD	22 min	Dilute-and-shoot	nicotine-cis-N-oxide, nicotine-trans-N-oxide, norcotinine, β-nornicotyrine, 1-methyl-3-nicotinoylpyrrolidine	Not validated LOD 0.01-0.03 µg/ml (not specified for each component)	
Westenberger *et al.*	2009	[194]	GC-MS/MS (without nicotine)	7.4 min	Only cartridges: methanol extraction	pseudooxynicotine, 1-methyl-3-nicotinoylpyrrolidine	No validation data mentioned	

The LC-MS/MS methods were validated according to the ISO guidelines. The accuracy and precision of the UV-detection method are significantly better compared to the tandem MS-methods because of its robustness. For MS/MS methods, the accuracy and RSD% are between 8-15% and 3-15%, respectively. The high dilutions, necessary for MRM quantification, imply that the working range compared to HPLC-DAD, is relatively small. The sensitivity (LOQ) was similar or slightly higher for the LC-MS/MS methods. Famele *et al*. was able to develop a LC-MS/MS method with the highest sensitivity for all related impurities (Table 4.4). The need for high sensitivity for the quantification of nicotine alkaloids in e-liquids is not pertinent in case nicotine is present in the e-liquids. The main criterion is the selectivity.

Furthermore, to increase the sample throughput and decrease the runtime, a differential ion mobility (DMS) method was proposed in order to obtain sufficient selectivity without a LC separation and a runtime of <1min [97].

3 FLAVOURINGS

The analysis of flavour components in e-liquids has become more important as soon as the potential harm, associated with inhaling these components, became clear. Next to propylene glycol and glycerol, flavourings make up the majority of the e-liquid content. One of the challenges of characterizing e-cigarette flavours is their wide chemical composition, which makes it difficult to develop one general method for all possible e-cigarette flavours. Another variable factor is the concentration range in which flavours are present in the e-liquids. Many of the methods so far describe analytical approaches for the qualitative screening, as quantitative determination of flavour requires exhaustive sample preparation and method validation that is more interesting for particular flavourings with a potential concern.

The characterization of flavours present in e-liquids is possible in a non-targeted approach. The most straightforward method is MS-screening. Hutzler *et al*. investigated 28 e-cigarettes using a GC-MS method with prior sample extraction using both acidic and alkaline extraction [88]. The identification of flavours was done by matching the spectra using the National Institute of Standards and Technology (NIST) library. The components are not additionally confirmed by comparing retention times with reference standards. Considering the high variety of flavouring components, it seems hardly likely to acquire all possible chemicals and confirm them individually. As an alternative for a more accurate untargeted screening, the use of high-resolution MS is recommended.

For quantification of flavourings, targeted approaches are applied with methods developed for a list of target flavourings. The target list can be short, including flavourings of concern such as diacetyl and

acetylpropionyl [131], which will be further discussed in Chapter 5. In some studies, the target list includes frequently used flavours, although it is unclear how the popularity of these flavours is determined [202]. Methods are reported in which flavours are simultaneously determined with other components, for example with the more harmful VOCs [151]. This is convenient as many flavour components are highly volatile and often analysed with the same (chromatographic) methodology i.e. GC-MS. Tierney *et al*. used GC-MS for the analysis of almost 90 flavours, mainly food flavourings [202]. The method, however, was not properly validated, leading to uncertainty about the reported quantities. Aszyk *et al*. developed and validated two complementary methods (GC-MS and LC-MS/MS) for the quantification of 88 flavours in e-liquids [110], [203]. Because of the wide variability in chemical groups of the flavours, resulting in a wide variability of volatility, the investigators opted to use both GC and LC. In many cases, the used flavours are high volatiles such as aldehydes, esters, diketons, etc. The non-volatile flavour group includes long-chain esters and ketons, pyrazines, etc. The same investigators also developed a GC-MS/MS method for a wide range of flavours, by injecting in splitless mode and setting the GC-oven temperature program to a high end temperature (300°C) in order to assure that also semi-volatile components could be determined as well [204].

The main issue with GC methods for e-liquid matrices is the associated matrix effect. To overcome this problem, matrix-matched calibration is used for the quantification to achieve an accuracy of <15%. The e-liquid matrix is viscous and greasy which may result in a faster deterioration of the GC-columns and deactivation of the interaction with the active groups. To overcome this issue, a proper sample preparation is needed to improve the life span of the column and to reduce the total number of cuts of the GC-column. So far, the dilute-and-shoot approach is applied for GC-methods, which may result in a high uncertainty of the quantitative results, though the use of a non-direct injection technique might overcome this problem. The use of headspace (HS) or thermal adsorption might eliminate the majority of the e-liquid matrix in order to avoid matrix effects and rapid deterioration of the GC-column.

Thus, to avoid problems with the e-liquid matrix, more appropriate sample preparation is needed and for accurate screening results this would be ideally in combination with HR-MS for the identification of flavours in e-liquids.

4 CONCLUSION

In general, the methods developed for e-liquids include GC-methods as the main target components are volatile. However, there are some challenges with GC-methods that can be overcome by the use of LC-methods. Most of the methods are targeted approaches while there is also a need for untargeted

screening methods, in order to detect unusual substances, which are more relevant for the screening of illicit additives. The methods developed for the analysis of nicotine and nicotine-related impurities are the best represented in scientific literature. From those, it is clear that the main challenges are selectivity/interference issues and the matrix effect. In summary, there is still a need for standardized and validated methods, specifically for e-cigarettes. The use of non-standardized methods also implies that the reported values of certain constituents are significantly different from one study to another. Of course, this might in part also be explained by the difference in quality of the e-liquids. However, it must be emphasized that comparison of different qualities of e-liquids can only be carried out in an appropriate way when the used methods have comparable sensitivity and reproducibility.

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PART II: ANALYTICAL ASSESSMENT OF E-CIGARETTES LIQUIDS

CHAPTER V - DEVELOPMENT AND VALIDATION OF A HS/GC-MS METHOD FOR THE SIMULTANEOUS ANALYSIS OF DIACETYL AND ACETYLPROPIONYL IN ELECTRONIC CIGARETTE REFILLS

As discussed in the introduction of the thesis, a frequently raised concern is the potential toxicity of certain flavours present in e-liquids, in particular diacetyl and acetylpropionyl. It is therefore important to be able to identify and quantify these compounds. Numerous analytical methods have been published for determining e-liquid compositions, but concerns exist with respect to the lack of analytical validation. Hence in this study, a new HS/GC-MS-based method has been developed for the screening and quantification of diacetyl and acetylpropionyl in e-liquids and subsequently fully validated using the 'total error' approach. As a proof of its applicability, the validated method was applied on a limited set of e-liquid samples, indicating that this methodology could be used for routine quality control analyses of e-liquids.

This chapter is based upon Barhdadi S., Canfyn M., Courselle P., Rogiers V., Vanhaecke T.* and Deconinck E.* (2017) Development and validation of a HS/GC-MS method for the simultaneous analysis of diacetyl and acetylpropionyl in electronic cigarette refills. *Journal of Pharmaceutical and Biomedical Analysis* 142: 218-224. IF_{2017} = 2.831. (*shared last authors).

1 INTRODUCTION

In recent years electronic cigarettes or e-cigarettes became increasingly popular. Market research studies in the media reported that in 2014 the world sales of electronic vapour products represented already $6 billion [1]. The Eurobarometer Tobacco survey of 2014 showed that e-cigarettes are equally used as nicotine replacement therapies to quit smoking, even though their efficacy still remains a point of discussion [2]. At the European level, the Tobacco related Products Directive 2014/40/EU (TPD) was recently implemented in the different Member States. According to the TPD, the e-cigarette is considered a tobacco related product. Since the TPD is a Directive and not a Regulation, individual Member States are allowed to regulate e-cigarettes in a more stringent way, for example as a medicinal product. In short, the TPD sets out the minimal quality and manufacture requirements for e-cigarettes together with some measures to mitigate potential health risks [3]. The use of flavours, however, has not been directly addressed, although it is a major discussion point between the industry, public health professionals and regulators. Indeed, not only may flavoured e-liquids with sweets-sounding brand names attract teenagers and young adolescents, concerns have also been raised with respect to the potential toxicity of certain flavours [4]. In fact, research on the toxicological profile of flavour additives in e-liquids and in inhaled vapours is scarce, yet essential as exemplified by

diacetyl [5]. This food additive has been widely used in e-liquids to generate a characteristic creamy and buttery flavour. Although diacetyl is mentioned in the Generally Recognized As Safe (GRAS) list of the Flavors and Extracts Manufacturers' Association (FEMA) and thus approved within certain limits for ingestion, scientific data show that the inhalation of diacetyl could be associated with lung diseases such as bronchiolitis obliterans [6]. As a reaction on these results, e-liquid manufacturers started to use the compound acetylpropionyl, also a α-diketon, as a substitute for diacetyl. However, it was recently shown that acetylpropionyl might also be a potential inhalation toxicant [7].

According to Directive 2014/40/EU, the use of diacetyl and acetylpropionyl in e-liquids is not explicitly prohibited nor restricted. Member States are, however, allowed to prohibit certain flavours if scientific evidence of potential health risks emerges [8]. In the UK and other Member States a ban of these compounds is already under discussion and even proposed in a draft guidance by the European Commission Working Group on notification of e-cigarettes and refill containers [9]. In view of the expected upcoming regulations, quality control analyses of e-liquids will gain more importance. Therefore the development of adequate and validated analytical techniques is highly necessary.

Several analytical methods suitable for the determination of diacetyl and acetylpropionyl in food and beverages, particularly in beer, have already been published. Typically, the vicinal diketons are first transformed to a more stable derivative and then analyzed. This can be done using high-performance liquid chromatography (HPLC) coupled to an ultraviolet (UV) detector or by gas chromatography coupled to mass spectrometry (GC-MS) to achieve a higher sensitivity [10], [11]. To assess workplace exposure, diacetyl and acetylpropionyl have also been determined in air samples. The method validated and issued by the Occupational Safety and Health Administration (OSHA) is based on the detection of a carbonyl−pentafluorobenzyl hydroxylamine derivative (PFBHA) with GC coupled to an electron capture detector (GC-ECD) [12]. It has been applied to analyze diacetyl concentrations in e-cigarette vapours [13]. To determine diacetyl and acetylpropionyl in e-liquids, an adapted version of the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) method for the determination of carbonyls in mainstream cigarette smoke was applied [14]. This method involved HPLC-UV with derivatization.

In this study a sensitive headspace (HS) GC-MS-based method is developed and validated. It enables the screening and quantification of diacetyl and acetylpropionyl simultaneously in an e-liquid matrix. As no derivatization is needed, the method is easier and less time consuming than existing methodology. It also provides the possibility of routine high throughput sample analysis.

2 MATERIALS AND METHODS

2.1 Standards and reagents

The reference standards diacetyl (2,3-butanedione) and acetylpropionyl (2,3-pentanedione) were purchased from Sigma-Aldrich (St. Louis, USA) and 1-butanol, used as an internal standard, was bought from Merck (Darmstadt, Germany). All standards had a purity grade of more than 97%. Analytical grade dimethylsulfoxide (DMSO) and sodium chloride were obtained from Merck (Darmstadt, Germany). Matrix components propylene glycol and glycerol were purchased from Merck (Schuchardt, Germany).

2.2 E-liquid samples

Twelve e-liquid samples were provided by the Belgium Federal Agency for Medicinal and Health Products (FAMHP) that were intended to be screened for the presence of nicotine. These samples were either obtained upon inspections of different vaping shops in Belgium in the period between 2013 and 2015 or were seized postal packages ordered by individuals through the internet. All samples were stored at ambient temperature and protected from light.

2.3 Sample preparation

2.3.1 Standard solutions

The calibration standards were prepared from a 10 mg/ml stock solution of both diacetyl and acetylpropionyl in DMSO. The stock solutions were stored in amber glass at 4°C. A separate 10 mg/ml stock solution in DMSO was prepared for the internal standard 1-butanol. The calibration solutions were obtained by diluting the diacetyl and acetylpropionyl stock solutions into the different concentrations 0.1 µg/ml, 0.25 µg/ml, 1 µg/ml, 2.5 µg/ml, 5 µg/ml and 10 µg/ml with DMSO as solvent in a 10 ml volumetric flask together with 1 g propylene glycol/glycerol matrix (70/30) and the internal standard at 5 µg/ml. After vortexing for 10 s, 300 µl was transferred into a 10 ml headspace vial to which 0.05 g of sodium chloride was added.

2.3.2 Validation formulations

The validation samples were prepared in a similar way as described above. A new 10 mg/ml stock solution was made of the reference standards. The validation samples were prepared at three concentration levels in triplicate. To prepare the validation samples, a propylene glycol/glycerol matrix was spiked with the reference standards of the analytes. An internet search revealed that the most common matrix ratio consists of 70% propylene glycol and 30% glycerol [15]. Therefore, 1 g of 70/30

propylene glycol/glycerol matrix was spiked with a 100 µg/ml standard working solution to obtain 5 ppm, 50 ppm and 100 ppm formulations in 10 ml and with the internal standard in a concentration of 5 µg/ml. Finally the solutions were mixed for 10 s and 300 µl was transferred into a 10 ml headspace vial to which 0.05 g of sodium chloride was added.

2.3.3 Sample preparation

Approximately 1 g of each e-liquid sample was weighted into a 10 ml brown flask and 500 µl of internal standard was added. This was further diluted to 10 ml with DMSO. After vortexing for 10 s, 300 µl was transferred into a 10 ml headspace vial to which 0.05 g of sodium chloride was added.

2.4 HS-GC/MS conditions

Method development and validation were performed on an Agilent 6890 N gas chromatograph coupled to an Agilent 5973N single quadrupole mass spectrometer and equipped with a G188A static headspace sampler (Agilent Technologies, Palo Alto, USA). The samples were contained in 10 ml sealed vials, which were placed in the autosampler oven to be heated and agitated in order to generate the headspace. The incubation temperature was maintained at 85°C with an equilibration time of 15min. The injector port was kept at 160°C, in split injection mode (split ratio 15:1), while the temperatures of the headspace loop and the transfer line were maintained at 100 and 120°C, respectively. The components were separated on a VF-5 ms (5% phenyl-95% methylpolysiloxane) capillary column of 60m with Ø 0.25 mm and film thickness of 0.25 µm and an integrated guard column of 10 m (#CP9013, Factor four, Agilent, California, USA). Helium carrier gas was used at a constant flow of 1.0 ml/min. The initial oven temperature of 55°C was held for 13 min, followed by a temperature ramp of 50°C/min to a final temperature of 250°C. The total run time was 16.90 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Temperatures of the ion source, the quadrupole, and the interface were set at 230, 150 and 280°C, respectively. The chromatograms of the initial screening were obtained in full scan mode from 25 to 400 m/z. For quantification, the mass spectrometer was operated in selective ion monitoring (SIM) mode (100 ms dwell times). The ions monitored for diacetyl were 43 and 86 m/z; for acetylpropionyl 43 and 57 m/z; for butanol (internal standard) 41 and 56 m/z. The first ion mentioned for each component was used for quantification and the second ion as a qualifier ion. The response was expressed by the ratio between peak areas of the analyte and the internal standard.

2.5 Method validation

Validation of the presented method was performed using accuracy profiles. This is a visual representation of the method-performance that integrates several validation parameters into one statistic i.e. trueness, precision and accuracy. The main difference between the classical validation approach and the total error approach, on which the accuracy profiles are based, is the use of a tolerance interval and the concept of total error.

The basic principle of the accuracy profiles procedure is that the fitness-for–purpose of a method is ensured by demonstrating that a defined percentage of the future results (β-expectation tolerance interval) will fall within certain predefined acceptance limits. To estimate this expected proportion of future observations, the total error of an analytical method needs to be considered. The total error is the difference between an observed result and the true value; it is the result of the systematic error (trueness) and the random error (repeatability and intermediate precision) of an analytical method. More information on the theoretical background of the total error approach and the equations used for the calculations in this paper, is described in [16]–[18].

For the validation of diacetyl and acetylpropionyl, the β-expectation tolerance intervals were set at 95%. Currently, there is no agreement on the acceptance limits to be used for e-liquids as is for active ingredients in pharmaceuticals (± 5%). For e-liquids an acceptance limit of ± 10% was regarded as acceptable considering the presence of matrix effects and the low concentration working range.

The validation samples (as prepared in 2.3.2) were analyzed in triplicate for five consecutive days. The calibration curves generated for each series were used to back-calculate the concentrations. Trueness, precision, and accuracy were determined for each concentration level. The accuracy profiles were built by plotting the concentration levels against the recoveries (%), including the acceptance limits and the upper and lower tolerance limits.

The other validation parameters assessed were selectivity, matrix effect, linearity of the calibration line, linearity of the results, limit of detection (LOD), limit of quantification (LOQ), and the recovery in similar matrices.

3 RESULTS AND DISCUSSION

3.1 Method development and optimization

The screening method for diacetyl and acetylpropionyl was developed as a part of a multicomponent screening of flavours in e-cigarette refills. Initial tests showed that the dilution solvent hexane interfered with relevant peaks. Therefore, DMSO and ethanol were included as dilution solvents. DMSO appeared to be most suitable for diacetyl and acetylpropionyl since, due to the delayed elution of the solvent peak compared to the target compounds, no interference was observed. For ethanol, the resolution was sufficient, however it was not chosen as dilution solvent because of practical considerations.

The headspace oven temperature was tested at high (135°C) and low (85°C) temperature. High headspace temperature is needed in case e-liquid components are difficult to evaporate. Both diacetyl and acetylpropionyl were sufficiently volatile to be detected at 85°C. To avoid the evaporation of other components present in the e-liquids, the low headspace oven temperature was chosen. Next, the GC temperature program was optimized. The initial hold time was kept as short as possible in order to shorten the total run time of the analysis. The initial GC oven temperature that resulted in the shorter retention time was 55°C. The sensitivity of the signal was further improved by adding 0.05 g sodium chloride to the vial. In Table 5.1, a short summary of all investigated parameters and optimization results are shown.

The method was first developed on a VF-624 ms (6% cyanopropylphenyl-94% dimethylpolysiloxane) capillary column (60 m×0.32 mm; 1.8 µm film thickness) and its performance was compared to that of a DB-5 ms (5% phenyl-95% methylpolysiloxane) capillary column (60m×0.25mm; 0.25µm). Both resulted in a good separation and resolution. The DB-5 ms capillary column was finally chosen because of the shorter retention times. The resolution of both components was comprised by transferring the method to the alternative capillary column. This issue was resolved by adjusting the split ratio and switching to constant flow modus. A chromatogram of diacetyl and acetylpropionyl in SIM modus is shown in Figure 5.1.

Table 5.1: HS/GC parameters optimized during method development. GC parameters in column (II) represent the final method parameters.

Optimization parameters	Evaluation criteria	I	II
Headspace-oven temperature		135°C	85°C
	Retention time diacetyl	14 min	14 min
GC temperature program Initial Time		9 min	13 min
	Response	Peak not detectable	Peak detectable
GC temperature program Initial Temperature		45°C	55°C
	Retention time diacetyl	14 min	11 min
Addition sodium chloride		1 g	0.05 g
	Response	No significant enhancement response compared to addition of 0.05g salt	Enhancement response compared to no addition of salt
Column stationary phase		624	VF-5
	Retention time diacetyl	11 min	7 min

3.2 Matrix effect

Recovery issues were encountered which led to the investigation of potential matrix effects. Although a number of analytical methods for e-liquids have been published, only a few mentioned potential matrix effects [19]. Most analytical methods, GC-MS and LC-MS, do not include any sample preparation step to clean up the matrix, which could interfere with the analytical testing [20].

Significant matrix effects were observed when, for the same concentration, the response obtained in the matrix was lower than the response of the standards, which is not uncommon with this kind of matrix. Indeed, matrix effects have previously been described for the analysis of cosmetics containing glycerol and propylene glycol [21]. A matrix effect was still observed when an internal standard was added. With the use of headspace, however, a part of the matrix never comes in contact with the chromatographic system. Therefore, the use of standard addition and preparation of calibration curves in a representative matrix were explored. Both options generated acceptable results but as standard addition is more labour-intensive and time-consuming, the quantification was proceeded using matrix-matched calibration curves.

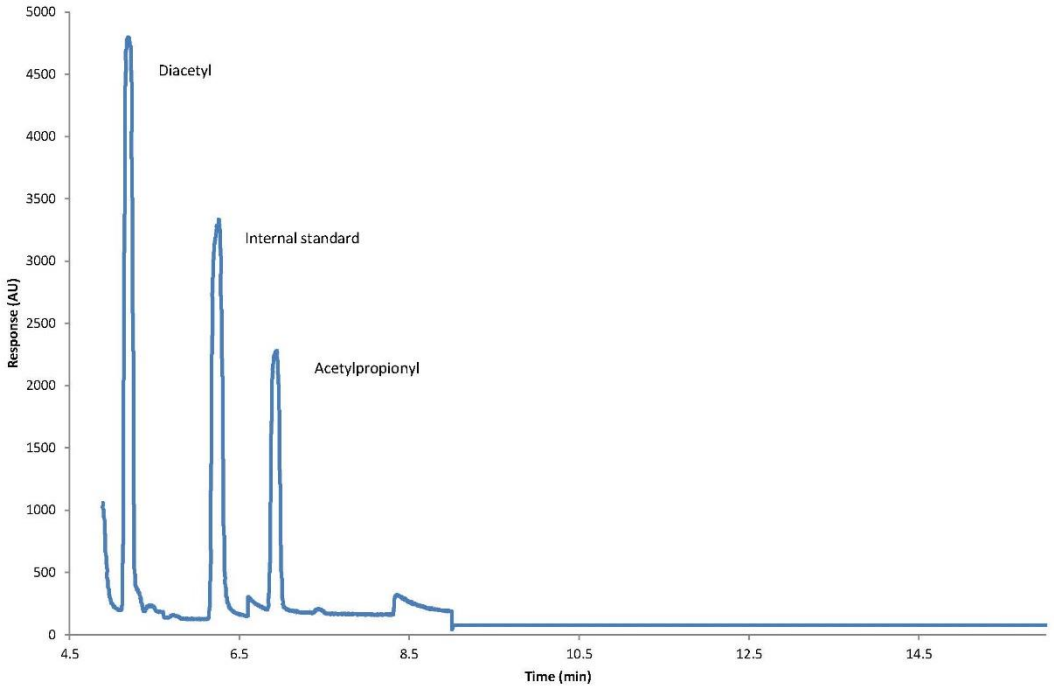


Figure 5.1: *Chromatogram of diacetyl and acetylpropionyl standards in propylene glycol/glycerol matrix at 0.5 ppm. The chromatogram was recorded in SIM mode. (AU = abundance units)*

3.3 Method validation

3.3.1 Selectivity and specificity

The selectivity of the method is important as, in first instance, it will be used for screening purposes. It was found that the matrix components of the spiked sample did not interfere with the detection of any of the target analytes. No interference was detected from additional investigated components which are likely to co-elute with the target analytes i.e. ethanol and ethyl acetate.

The specificity of the quantification method is ensured by monitoring the specific ions of diacetyl and acetylpropionyl in SIM modus.

3.3.2 Linearity of the calibration curve

The calibration curve is established from five calibration points in a concentration range of 0.1-10 µg/ml by applying the least square linear regression. The linearity was confirmed using R^2 values, the quality coefficient (QC) and the lack of fit test. The latter showed that the calibration curves describe the observed linear relationship within the chosen concentration range. The R^2 and QC values are summarized in Table 5.2 with all R^2 values being above 0.999 and the QC values below 4%.

Table 5.2: Validation of the linearity of the calibration line for diacetyl and acetylpropionyl: R^2 - and QC values are obtained for all the calibration lines obtained during method validation for diacetyl and acetylpropionyl.

	R^2	QC (%)
Diacetyl	[0.9990, 1.0000]	[0.53, 3.71]
Acetylpropionyl	[0.9991, 0.9998]	[1.51, 3.80]

3.3.3 Method linearity

The linearity of the results, demonstrated as the relationship between the measured concentration and the theoretical concentration, is linear with R^2-values above 0.9999 for both components.

3.3.4 Trueness and Precision

The trueness is the closeness of agreement between the average value of a series of measurements and the true value, in this case the exact known concentration of the validation samples. It estimates the systematic error of an analytical method and is expressed as a relative bias at each concentration level. As shown in Table 5.3, the relative bias for both components was well below 10% with a maximum relative bias of 3.04%. Consequently, the validation requirements are fulfilled.

The precision is the closeness of agreement between the values obtained from repeated measurements. It estimates the random error of the method and is expressed using relative standard deviation (RSD). For each concentration level, the repeatability was obtained from the variability of the triplicate measurements. The intermediate precision was investigated as well for the time-dependent variability of the method. The results are displayed in Table 5.3. The highest value was seen for diacetyl with a repeatability and intermediate precision of 3.52% which was considered acceptable, as also confirmed by the accuracy profiles (see further).

Table 5.3: Trueness, precision and accuracy of the analytical method for diacetyl and acetylpropionyl. (RSD: relative standard deviation)

	Trueness			Precision						Accuracy		
	Relative bias (%)			Repeatability (RSD)			Intermediate precision (RSD)			β-expectation tolerance limit (%)		
Concentration level (ppm)	5	50	100	5	50	100	5	50	100	5	50	100
Diacetyl	-0.96	-1.68	-0.59	1.59	3.52	2.28	2.80	3.52	2.71	[-9.38 ; 7.46]	[-9.69 ; 6.34]	[-8.11 ; 6.94]
Acetylpropionyl	-3.04	-3.00	-1.42	2.18	2.06	1.58	2.29	2.12	2.26	[-8.22 ; 2.15]	[-7.78 ; 1.78]	[-7.31 ; 4.48]

3.3.5 Accuracy and LOQ

Based on the obtained trueness and precision of the method, the β-expectation tolerance intervals, representing the accuracy of the method, were calculated. Accuracy takes the total error associated with each measurement into account. The accuracy profiles of diacetyl and acetylpropionyl are presented in Figure 5.2 and the β-expectation tolerance intervals are given in Table 5.3. The accuracy profiles show that the β-expectation tolerance intervals do not exceed the acceptance limits of ± 10%, which means that 95% of the future measurements will be included in the [-10%, 10%] bias limits. Therefore, this method is considered suitable for the intended purpose.

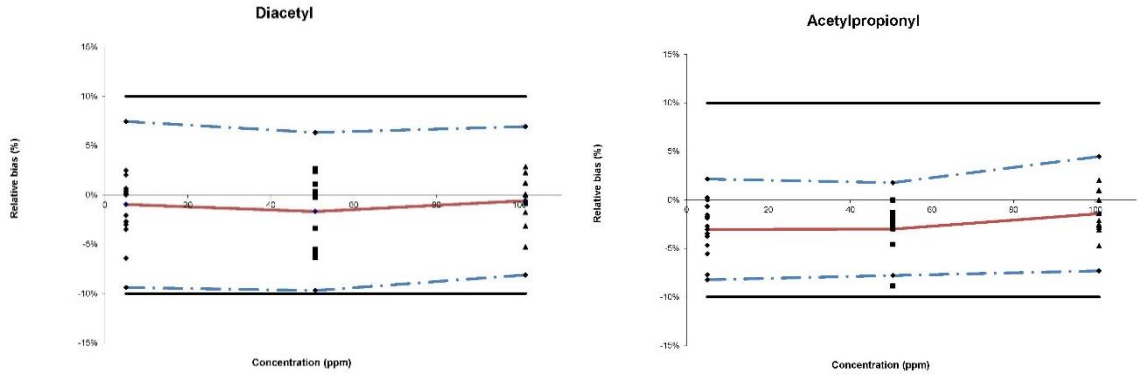


Figure 5.2: *Accuracy profiles obtained for (a) diacetyl and (b) acetylpropionyl with β set at 95%. Legend: Relative bias (–), upper and lower β-expectation tolerance limits (– •), upper and lower acceptance limits set at 10% (–), relative back-calculated concentrations per spiking level (◆▲■).*

The LOQ is also acquired from the accuracy profiles and is defined by the concentration where the β-expectation tolerance interval crosses the acceptance limit. If the β-expectation tolerance intervals do not cross the acceptance limits, the lowest tested concentration level can be considered as the LOQ, as is the case in this study. The LOQ for diacetyl and acetylpropionyl was set at 5.08 ppm and 5.05 ppm, respectively.

3.3.6 LOD of the screening method

The sensitivity of the screening method was assessed by determining the LOD. The LOD was estimated as the concentration with a signal-to-noise ratio of at least three as recommended by the International Council for Harmonisation (ICH) guidelines [22]. Samples with known decreasing concentrations were analyzed to empirically determine the LOD. The LOD was estimated based on the extracted ion chromatograms because of the decreased background noise. The obtained results for diacetyl and acetylpropionyl were 3.16 ppm and 7.86 ppm, respectively. The obtained LOD for acetylpropionyl is higher than the LOQ, as the LOD was estimated using the screening method in total ion current (TIC) mode, while the LOQ was determined using the quantification method in SIM mode. Today, however,

it is not possible to recommend a required sensitivity for the analytical methods intended for e-liquid analysis, as currently there are no inhalation toxicity limits available for diacetyl and acetylpropionyl in e-cigarettes.

3.3.7 Recovery

The method was validated using spiked 70/30 propylene glycol/glycerol matrix. To evaluate the method for other e-liquid matrix compositions, recoveries were determined for a 100% propylene glycol, 100% glycerol and 50/50 propylene glycol/glycerol matrix spiked at the intermediate concentration level in triplicate. The matrix used for the matrix-matched calibration for all matrices was the 70/30 propylene glycol/glycerol matrix. The results are shown in Table 5.4. The obtained recoveries were all between 90% and 110%. Since the matrices used in the recovery study cover the whole range of different propylene glycol and glycerol e-liquid matrix compositions, the method is suited for the analysis of diacetyl and acetylpropionyl in all propylene glycol/glycerol e-liquids.

Table 5.4: Recovery of diacetyl and acetylpropionyl at 10 ppm for different ratios of propylene glycol and glycerol in the matrix (analyzed in triplicate).

Compounds	Recovery (%)			
	Propylene glycol	30/70	50/50	Glycerol
Diacetyl	99.36 ± 2.44	106.24 ± 3.07	104.26 ± 0.72	106.25 ± 1.02
Acetylpropionyl	90.96 ± 0.38	96.70 ± 1.97	96.22 ± 0.71	100.02 ± 1.41

3.4 Screening and quantification of e-liquid samples

The validated method was used for the simultaneous determination of diacetyl and acetylpropionyl in a subset of e-liquid samples. The samples were first screened in full scan mode for diacetyl and acetylpropionyl. Their presence was confirmed by comparing the obtained MS-spectrum to the NIST library. Additional confirmation of the components was based on the relative retention times and the ratio of the prominent ions in the mass spectrum. The maximum tolerated error for the relative retention time was 0.05 min. Out of the 12 selected e-liquids that were screened, two samples tested positive for diacetyl (vanilla and pina colada flavour) and another two for both diacetyl and acetylpropionyl (café noisette and coconut flavour). The positive samples were subsequently analyzed with the method in SIM modus for quantification. Here, only the café noisette and coconut flavoured e-liquids appeared to contain measurable amounts of diacetyl (98.84 µg/g and 6.04 µg/g, respectively)

and acetylpropionyl (10.86 µg/g and 8.36 µg/g, respectively). A chromatogram of the café noisette flavour e-liquid is shown in Figure 5.3.

Due to the rapid evolution of the e-liquid market, these samples may not be representative anymore for the current market. The analysed e-liquids were sampled in 2013-2015. In this period, the awareness of potential harm related to the use of diacetyl and acetylpropionyl as flavours was low. Nowadays, manufacturers present their e-liquids as diacetyl-free. However, as observed before, label claims of e-liquids do not always correspond with the actual composition, and thus proper quality controls (QC) will remain necessary [23].

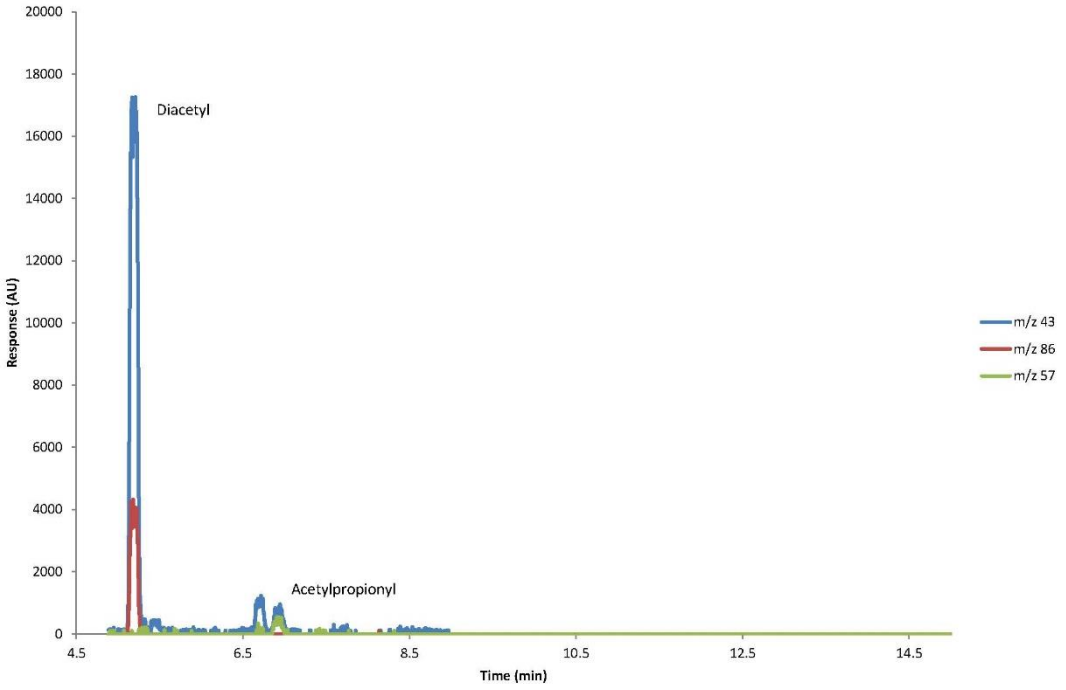


Figure 5.3: *Extract ion chromatogram recorded in full scan of the investigated sample café noisette containing both diacetyl as acetylpropionyl. Diacetyl: quantifier = 43 m/z, qualifier = 86 m/z. Acetylpropionyl: quantifier: 43 m/z, qualifier = 57 m/z (AU = abundance units).*

To date, there is no scientific consensus on a maximum allowed level of diacetyl and acetylpropionyl in e-liquids. Difficulties encountered relate to the fact that the amount of diacetyl and acetylpropionyl present in the vapours can be highly variable and that different inhalation exposure scenarios exist (e.g. heavy *versus* light smoker). The suggestion to use exposure limits reported by the National Institute for Occupational Safety and Health (NIOSH) or the Scientific Committee on Occupational Exposure Limits (SCOEL) has raised some resistance. Indeed, as also pointed out in earlier publications, these limits are not suitable to establish safe exposure limits for the general public [13], [24]. Moreover, the inhalation pattern during normal breathing differs from the intentional inhalation which occurs during vaping of e-cigarettes. Therefore, it is assumed that the allowable diacetyl and

acetylpropionyl concentrations for the general public will be much lower than the proposed occupational exposure limits.

4 CONCLUSION

A HS/GC-MS-based method to screen and quantify diacetyl and acetylpropionyl simultaneously in e-liquids was developed and successfully validated using accuracy profiles. The proposed method uses no prior derivatization. Therefore, testing is easier and less time-consuming. Because of the use of headspace, no extensive clean-up is needed. Without the need for direct injection of the propylene glycol/glycerol matrix, the method is also less damaging for the column and the instrument. The matrix effect caused by the matrix components in the headspace is bypassed by the use of a matrix-matched calibration. The validation results show that the method is fit-for-purpose. The screening method detects concentrations above 3.16 ppm and 7.86 ppm for diacetyl and acetylpropionyl, respectively. Quantification of diacetyl and acetylpropionyl in e-liquid samples is possible in the working range of 5 µg/ml – 100 µg/ml with ± 10% accuracy. This methodology could be used for routine quality control analyses of e-liquids.

CHAPTER VI - A SIMPLE DILUTE-AND-SHOOT METHOD FOR SCREENING AND SIMULTANEOUS QUANTIFICATION OF NICOTINE AND ALKALOID IMPURITIES IN ELECTRONIC CIGARETTE REFILLS (E-LIQUIDS) BY UHPLC-DAD

Besides the presence of potential harmful flavourings such as diacetyl and acetylpropionyl, the other most encountered quality and/or safety problem of e-liquids is the nicotine label discrepancy and the presence of nicotine impurities in e-liquids. From a regulatory point of view, the analysis of nicotine is the most straightforward quality indicator for e-liquids. Different methods have been published to measure nicotine and its impurity levels, but the majority of them use a targeted LC-MS/MS approach. There is, however, a need for more robust quantification methods that are easy to implement in most control (industrial and governmental) laboratories (see Chapter IV). Therefore, in this chapter, the development and validation of a simple dilute-and-shoot UHPLC-DAD method for the simultaneous quantification of nicotine and its alkaloid impurities in e-cigarette refills is set up. The method validation is carried out according to the "total error" approach and is in accordance with the validation requirements of ISO-17025. Additionally, the interference between the target components and a number of popular flavouring components such as vanillin, maltol, ethylacetate, etc. has been investigated. Also, the robustness of the method has been assessed.

This chapter is based upon: Barhdadi S., Desmedt B., Courselle P., Rogiers V., Vanhaecke T.* and Deconinck E.* (2019) A Simple dilute-and-shoot method for screening and simultaneous quantification of nicotine and alkaloid impurities in electronic cigarette refills (e-liquids) by UHPLC-DAD. *Journal of Pharmaceutical and Biomedical Analysis* 169: 225-234. IF_{2018} = 2.983. (*shared last authors).

1 INTRODUCTION

As shown by the increasing sales of the past years, the e-cigarette has become widely popular [25]. The e-cigarette market is characterised by a quick product evolution starting from cig-a-like pens over replaceable cartridges to refillable liquids (e-liquids). Nowadays, refillable e-liquids are the most commonly used in Europe [26]. In most European countries, the e-cigarette became readily available although it is not yet clear whether it can be used as a smoking cessation tool, therefore more clinical evidence is needed [27].

The requirements for e-cigarettes on the European market are regulated by the Tobacco Product Directive (TPD)[3]. The directive stipulates the prohibited use for minors, the labelling and packaging requirements, the use in public spaces and marketing limitations. However, regarding the allowed ingredients, the TPD only provides limited information. The TPD explicitly forbids carcinogenic, mutagenic and reprotoxic substances (CMR) and products associated with energy and vitality such as

caffeine, taurine and vitamins. Besides the maximum allowed nicotine concentration limit of 20 mg/ml, no other requirements are mentioned. Furthermore, there are no requirements set regarding the purity of the ingredients. These gaps in the Directive could have an impact on the quality and therefore the safety of the e-liquids [28]. Quality standards for e-liquids are thus highly needed, more so because of the recent discovery of counterfeit 'e-liquids' [29]. A CEN/ISO workgroup is currently focusing on standardized methods for the analysis of e-liquids, meeting the urges for harmonization of analytical methods for these new tobacco products [30].

One of the most investigated ingredients in e-liquids is nicotine. The amount of nicotine and compliance with the labelled nicotine concentration is an important quality parameter. Studies have shown that in a number of cases the nicotine concentration found in e-liquids deviates from the nicotine concentration claimed on the packaging. *Vice versa*, nicotine has also been found in so-called zero-liquids, probably due to poor cleaning procedures or lack of appropriate labelling practices [31]–[33]. Yet, low quality e-liquids are not only characterised by nicotine mismatch or mislabelling due to poor manufacturing conditions, but also by the presence of impurities.

One group of these impurities are the nicotine related impurities that consist of nornicotine, anabasine, anatabine, myosmine, cotinine, β-nicotyrine and nicotine-N-oxide (Figure 3.3). These minor alkaloids are known to contribute to the organoleptic properties of cigarette smoke in tobacco cigarettes and are also traditionally used as an indicator of tobacco quality [34]. The nicotine-alkaloïd presence in e-liquids can have two origins. First, the nicotine used in the e-liquids is extracted from tobacco plants and, depending on the method of extraction, nicotine related impurities are extracted as well [35]. Hence, e-liquids may contain these alkaloid impurities when tobacco extracts have been used to obtain a tobacco flavour [36]. Secondly, the nicotine alkaloids can also be formed due to degradation of nicotine. Thus, storage is also an important factor to take into account.

So far, several analytical methods for the analysis of nicotine have been developed [33], [36]–[42]. The quantification of nicotine is easily done by UV-detection because of its chromophore. Furthermore, analysis with GC is feasible due to its volatility [43]. More relevant, however, is the simultaneous analysis of nicotine and its related alkaloids. In this case, GC is not eligible because of the thermally unstable alkaloid nicotine-N-oxide [44]. In addition, a clean-up is needed since the injection of the e-liquid matrix might cause an accelerated deterioration of the capillary column due to contamination with propylene glycol. The latter deactivates the active groups of the column which results in column bleeding, decreased sensitivity and high background noise [45]. The most prominent technique used so far is targeted LC-MS/MS in multiple reaction monitoring mode, which displays a much higher specificity than obtained through UV-detection [41], [42], [44], [46]–[48]. The presence of other

flavours can easily be circumvented with the targeted approach. LC-MS/MS, however, is more susceptive to matrix effects due to interference with propylene glycol and glycerol. The LC-MS/MS methodology used for the quantification of nicotine alkaloids are typically dilute-and-shoot methods. To eliminate the matrix effect of propylene glycol and glycerol, high sample dilutions and/or expensive deuterated internal standards are used (see Chapter IV for more detailed information about previously reported quantification methods on nicotine and minor alkaloids in e-cigarettes).

In order to avoid the use of expensive internal standards with MS detection, we suggest the use of UV for simultaneous detection and quantification of nicotine and its related impurities. We opted for LC with UV-detection because of its robustness and lower cost. Furthermore, this technique is available in nearly every quality control laboratory. The challenge with UV-detection is, as mentioned before, the putative interference of flavour compounds. Therefore, establishing the selectivity of the method is necessary. To ensure that the method is suitable for the intended purpose, it is also validated according to International Conference of Harmonisation (ICH) guidelines using accuracy profiles [33]. To further proof its applicability, extra e-liquids samples were analysed with the UHPLC-DAD method and the results were compared to those obtained by a validated targeted LC-MS/MS screening method.

2 MATERIAL & METHODS

2.1 Standards and reagents

The standards of nicotine, cotinine, anabasine and myosmine and boric acid were purchased from Sigma Aldrich (St. Louis, USA). Nornicotine and β-nicotyrine standards were bought from Toronto Research Chemicals Inc. (Toronto, USA). Nicotine-N-oxide and anatabine came from Cayman Chemical (Michigan, USA). The matrix components propylene glycol and glycerol were purchased from Merck (Schuchardt, Germany). The components used to reconstitute a flavoured e-liquid sample were all purchased from Sigma Aldrich (St. Louis, USA). These include diacetyl, acetylpropionyl, maltol, ethylmaltol, vanillin, ethylvanillin, citral, limonene, cinnamaldehyde, transcinnamaldehyde, benzaldehyde, carvone, pulgeone, damascenone, methylcyclopentone, methylcinnamate, methylaminobenzoate and methylmyristate. The solvents acetonitrile, methanol, acetone and ethylacetate were HPLC-grade and were purchased from Biosolve (Valkenswaard, The Netherlands). Concentrated ammonia 28-30% was obtained from Merck (Darmstadt, Germany). Water was obtained using a milliQ-Gradient A10 system (Millipore, Billerica, USA).

2.2 Sample preparations

2.2.1 Preparation of the calibration standards

The calibration standards were prepared from a 10 mg/ml stock solution in methanol. The stock solutions were stored in amber glass at 4 °C for maximum 1 week. For nicotine, calibration solutions were obtained by diluting the stock solutions with water in order to cover a concentration range from 0.5 to 20 µg/ml. The calibration range for each nicotine alkaloid impurity is given in Table 6.1.

Table 6.1: The nominal concentration range of the nicotine and related impurities calibration standards used for the method validation and analysis of e-liquid samples and in the validation samples with 70/30 propylene glycol/glycerol matrix. The actual concentrations might differ from the nominal concentration according to the weighted amount of standard. The validation samples are diluted 1:10 prior to analysis.

Component	Calibration samples	Validation samples		
	Calibration range (µg/ml)	Level 1 (µg/ml)	Level 2 (µg/ml)	Level 3 (µg/ml)
Anabasine	0.1 - 2.0	2	5	20
Anatabine	0.1 - 2.0	2	5	20
β-Nicotyrine	2.0 - 10.0	20	50	100
Cotinine	0.1 - 2.0	2	5	20
Myosmine	0.1 - 2.0	2	5	20
Nicotine	0.1 - 25.0	5	50	200
Nicotine-N-oxide	0.1 - 2.5	2	5	25
Nornicotine	0.1 - 10.0	5	50	100

2.2.2 Preparation of spiked matrix validation samples

In the case of e-liquids, a representative matrix is difficult to reproduce. In many cases a simplified matrix is used i.e. a mixture of propylene glycol and glycerol. The ratios of the components usually vary with 70/30 and 60/40 as the most often used ratio. This simplified matrix is appropriate to evaluate matrix effects and to validate accuracy and reproducibility of a method. Hereto, a propylene glycol/glycerol matrix was spiked with the reference standards of the analytes. Therefore, 1 g of 70/30 propylene glycol/glycerol matrix was spiked with the standards and further diluted 10x prior to injection. The mixture of propylene glycol/glycerol was spiked daily with the mixtures of the analytes from a standard working solution to obtain three concentration levels for each analyte. Working solutions were prepared daily, as well as the calibration curve for the quantitative analysis. The validation samples were prepared in triplicate at three concentration levels (Table 6.1). During 5 days,

samples were prepared at three spiking levels in triplicate. Thus, in total 9 samples were prepared every day together with the calibration curve for the quantitative analysis.

2.2.3 Preparation of matrix validation samples spiked with flavourings

For the validation of the selectivity, i.e. the investigation of interference by other components, more realistic samples are needed. A matrix representative for real e-liquid samples is almost impossible with all the different e-liquid flavour components that are available on the market. Therefore, a 'flavoured'-spiked matrix was prepared containing a selection of 19 commonly used flavouring components of different chemical classes regularly found in e-liquids. These included diacetyl, acetylpropionyl, maltol, ethylmaltol, vanillin, ethylvanillin, citral, limonene, cinnamaldehyde, transcinnamaldehyde, benzaldehyde, carvone, pulgeone, damascenone, methylcyclopentone, methylcinnamate, methylaminobenzoate, methylmyristate and ethylacetate. These 19 flavours were added at a final concentration of 1% in different combinations to a propylene glycol/ glycerol matrix.

For the assessment of the selectivity of the UHPLC-DAD method, the validation samples mentioned above were analysed to control for co-elution of these flavourings with the target components.

2.3 Equipment and chromatographic conditions

2.3.1 Quantification method of nicotine and alkaloid impurities (UHPLC-DAD)

The analyses were conducted on an Acquity UPLC™ system (Waters, Milford, USA) equipped with a photodiode array detector with a Waters Acquity BEH RP18 2.1 mm × 100 mm, 1.7 µm column and a Van Guard BEH pre-column (2.1 mm × 100 mm, 1.7 µm) to prolong the lifespan of the column. The gradient consisted of a 0.010 M ammonium borate buffer of pH 9.0 as mobile phase A and acetonitrile as mobile phase B. The gradient conditions are shown in Table 6.2. Acetonitrile was used as the strong needle wash solvent and 95% water and 5% acetonitrile as the weak needle wash solvent.

Table 6.2: The chromatographic parameters of the UHPLC-DAD and UHPLC-MS/MS methods to analyse nicotine and its related impurities.

	UPLC-DAD method			**UPLC-MS/MS method**		
Column	Acquity UPLC BEH C18 (Waters, 1.7µm, 100mm, 2.1mm) with Van Guard BEH C18 pre-kolom (1.7µm, 2.1mm, 5 mm)			Acquity UPLC BEH C18 (Waters, 1.7µm, 100mm, 2.1mm)		
Mobile phase	A) 10 mM ammoniumborate pH 9.0 B) 100% ACN			A) 0.1% ammonia in water B) 0.1% ammonia in ACN		
Gradient	Time (min)	Mobile phase A % V/V	Mobile phase B %V/V	Time (min)	Mobile phase A % V/V	Mobile phase B %V/V
	0 -1	95	5	0 -6	95	5
	1 -7	75	25	6-6.5	75	25
	7-9	75	25	6.5-7	95	5
	9-9.5	95	5			
	9.5-11	95	5			
Flow	0.4 ml/min			0.5 ml/min		
Injection volume	10 µl			10 µl		
Detection	254 nm, 234 nm and 285 nm			MRM transitions (Table 6.3)		
Column temperature	30°C			45°C		

The gradient had a flow of 0.4 ml/min. The injected volume was 10 µl in full loop modus for high reproducible results. Sample temperature was set at 10°C and the column temperature at 30°C. The wavelength used for the quantification of nicotine and most alkaloid impurities was 261 nm with the exception of myosmine and β-nicotyrine for which 234 nm and 285 nm were used, respectively.

2.3.2 Targeted-screening method of nicotine alkaloid impurities in multiple reaction monitoring (MRM) mode (UHPLC-MS/MS)

To proof the applicability of the UPHLC-DAD method, the analysis of commercial e-liquid samples was carried out using a LC-MS/MS screening method for the identification of the target components. A standard Waters Acquity ultra performance liquid chromatographic system (UPLC, Waters Corp., USA) was coupled with a Xevo TQ mass spectrometer (Waters Corp., USA). Solvent A consisted of 0.1% NH_4OH in water, while solvent B consisted of 0.1% NH_4OH in acetonitrile. The chromatographic separation was performed on an Acquity BEH C18 2.1 × 100 mm, 1.7 µm column (Waters Corp., USA). The injection volume was 10 µl (full loop modus), the flow rate 0.5 ml/min and the column temperature was maintained at 45°C. Total run time (including regeneration time of the column) was 7 min. The chromatographic conditions are shown in Table 6.2. The analytes were measured in positive electrospray ionization (ESI) mode. The monitored MRM transitions (two for each analyte) and the compound specific parameters can be found in Table 6.3. The cone voltage was set at 30 V for all target components. Capillary voltage was set a 3.5 kV, desolvation temperature was 350 °C. Desolvation and cone gas flow were set to 650 and 50 l/h, respectively. CID (Collision Induced Dissociation) was performed using helium as collision gas.

The screening method was validated for the intended purpose according to the validation guidelines [49], [50]. The obtained screening detection limit (SDL) was 50 ng/ml. The SDL is the lowest concentration for which it has been demonstrated that a given analyte can be detected in at least 95% of the samples. The specificity of the MRM method was assured by applying a minimum of 5 identification points (IP) in the method. Each component was detected by its relative retention time (1,0 IP), one precursor (1,0 IP) and two daughter ions (2 x 1,5 IP). A target component was identified correctly when the following criteria were met: max 5 % deviation from the retention time; the ratio of the two fragment ions differed not more than 30%; the S/N ratio was larger than 10. In Figure 6.1 a representative chromatogram is shown of an analysis of the nicotine impurities reference standards with the targeted screening method.

Table 6.3: Monitored MRM transitions of the LC-MS/MS method for the analysis of nicotine and its related impurities with their specific MS-parameters. (m/z = mass-to-charge ratio)

	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Anabasine	163	118	15
	163	146	15
Anatabine	161	107	15
	161	144	15
β–Nicotyrine	159	117	20
	159	144	20
Cotinine	177	118	20
	177	146	20
Myosmine	147	105	20
	147	130	20
Nicotine	163	117	20
	163	130	20
Nicotine-N-Oxide	179	117	25
	179	130	25
Nornicotine	149	117	20
	149	130	20

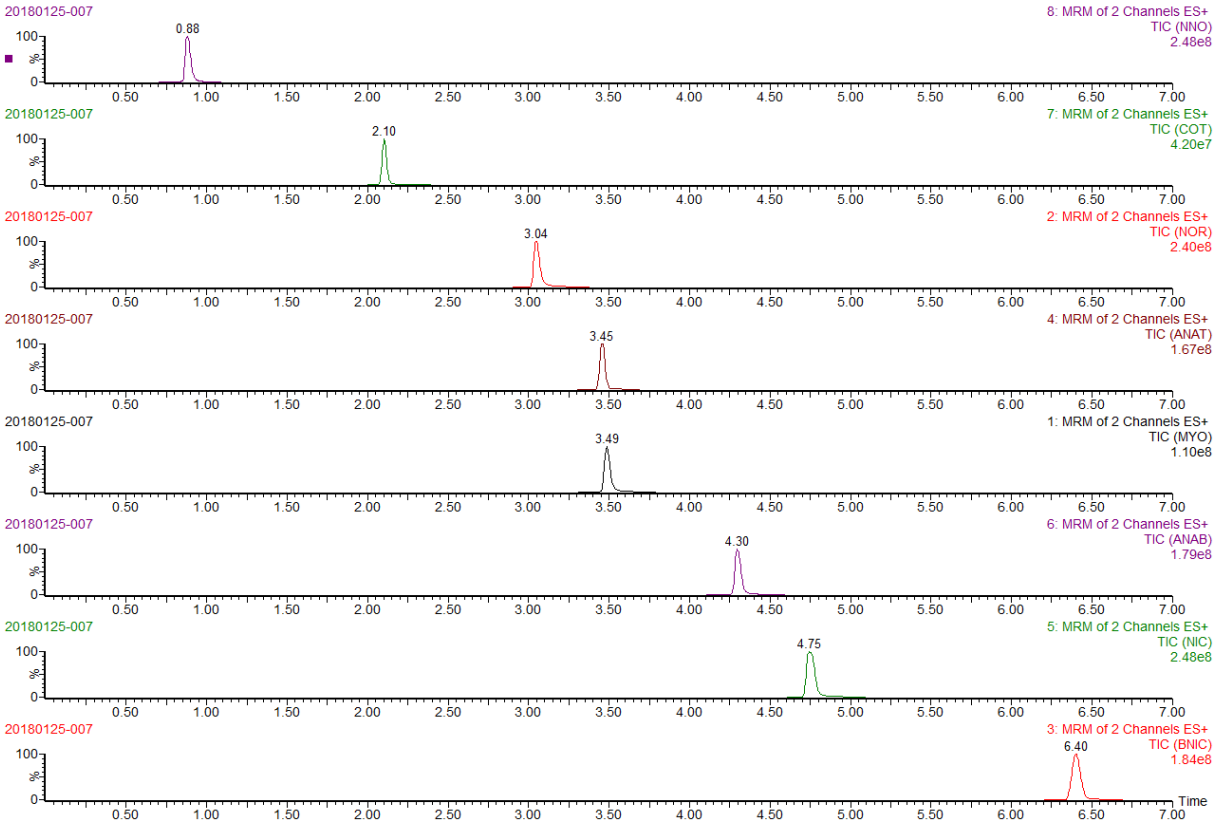


Figure 6.1: *Representative LC-MS/MS chromatogram of the target components with as response the sum of both selected MRM transitions.*

2.4 Method validation

Validation of the UHPLC-DAD method was performed using accuracy profiles. This is a visual representation of the method-performance that integrates several validation parameters i.e. trueness, precision and accuracy, into one statistic. For the method validation, the β-expectation tolerance intervals were calculated at 95%. Currently, there is no agreement on the acceptance limits to be used for e-liquids. Considering the wide concentration range, an acceptance limit of ±10% for nicotine and ±20% for the impurities was therefore regarded as acceptable.

The validation samples were analysed in triplicate for five consecutive days. The nicotine impurities were validated simultaneously in one series and separately from nicotine to avoid potential contamination from impurities of the nicotine standard. Trueness, precision, and accuracy were determined for each concentration level. The accuracy profiles were built by plotting the concentration levels against the recoveries (%), including the acceptance limits and the upper and lower tolerance limits. Besides the accuracy and the total error of the method, the selectivity of the method is important to establish and to validate. As mentioned before, the main issue with e-liquids is the potential interference of the matrix components such as flavourings. The other validation parameters assessed were linearity of the calibration line, linearity of the results, limit of detection (LOD), limit of quantification (LOQ), and the recovery in similar matrices.

The robustness of the UHPLC-DAD method was investigated separately after completing the fit-for-purpose validation procedures, to determine the allowed variability of method parameters without influencing the validity of the method. The test was performed by a three-factor three-level full factorial design [40]. The investigated factors were the column temperature and the pH and molar concentration of the ammonium borate buffer. The response was the resolution between the critical pair myosmine and anatabine. The values were chosen to cover typical errors that could occur. The buffer pH of the mobile phase was varied ± 0.2 of the validated value (pH=9.0). The selected buffer concentrations of the mobile phase varied 2 mM from the method value (10 mM) and the column temperature varied ±5°C from the method value (30°C). The effect of each factor was calculated for its significance at a 5% level using an ANOVA analysis.

2.5 Proof of applicability

Ten extra e-liquid samples were collected from two different channels; five samples were bought in Belgian vape shops and five samples were obtained from the internet. All samples were stored at 4°C and protected from light. Approximately 1 g of the acquired e-liquid samples was weighed into a 10 ml brown flask, thus a 1:10 dilution was used for the analysis of the alkaloid impurities. For the

quantification of nicotine, the 1:10 dilutions were out of range for some samples and a higher dilution factor was necessary. Samples were diluted with water until a nicotine concentration within the interval of the calibration line was obtained. For the LC-MS/MS analysis, these final solutions were filtered with 0.2 µm PTFE (polytetrafluoroethylene) filters prior to injection. Samples were analysed in duplicate. The nicotine concentration of the samples is expressed as mass of nicotine per volume e-liquid. From a practical point of view, working on a volume based manner such as pipetting of e-liquids was considered not precise enough because of the viscosity of the e-liquids. Therefore, a weigh-based approach was chosen. However, in order to be able to recalculate the nicotine concentration from a weight-to-weight unit to a weight-to-volume unit, the density had to be determined. Hence, a DM40 density meter (Mettler Toledo, Colombus, US) was used to determine the density of every e-liquid in the sample set.

3 RESULTS AND DISCUSSION

3.1 Method development

The European Pharmacopoeia (Ph.Eur.) method described in the monograph of nicotine for the analysis of the related impurities was used as starting point for the method development [51]. To obtain a faster analysis time, the original HPLC method was transferred to UHPLC. As such, we were able to decrease the run time from 40 min to 11 min. The resolution of the critical peak pair myosmine and anabasine was less than 2 and therefore not baseline separated when the original aqueous mobile phase consisting of 25 mM ammonium acetate pH 10 was used. Additionally, as a result of a precipitation reaction between the mobile phase; ammonium acetate and acetonitrile, a blockage of the UHPLC pump was caused. The first option was to change the organic mobile phase. However, methanol was not strong enough to elute all the target components. Thus, the aqueous mobile phase was changed. As an alternative, ammonium borate in different molar concentrations was used and the pH was screened until baseline separation was obtained for the critical peak pair. As the ammonium borate buffer consists of borate salts potentially affecting the column, an extra pre-column was used to prolong its lifetime. Matrix effects were visually inspected through chromatogram overlays of the target components with and without the presence of e-liquid matrix in order to check whether the use of internal standards was necessary. There was no indication of a matrix effect as the chromatogram overlay did not show any response enhancement or suppression of target components and the recovery was well within the accuracy limits. Thus, no internal standard was needed for quantification with UHPLC-DAD.

3.2 Method validation

3.2.1 Selectivity and specificity

The specificity of the quantification method for nicotine and its related impurities is low compared to the targeted MS/MS screening method. The UV-spectra of the target components resemble one another because of the structural similarity of the related impurities. The identification of the components is therefore based on the determination of the retention times and the UV-spectral matching with a reference standard.

Screening of the prepared flavoured samples spiked with the target components showed, that there was no interference between the selected flavours and the related impurities (Figure 6.2). This does not imply that there are no interferences of the target components with other flavours/additives. There are more than hundreds of chemicals present in e-liquids. However, not all these components are able to interfere in DAD-detection as there are certain conditions to be met before they could pose a problem; 1) components need to have a chromophore (not always the case for highly volatile aldehydes) and 2) the ability to be separated on a C18 column with a high pH buffer – meaning that components with an acidic carboxylgroup would already be ionized, thus not separated on the column. Hence, basic components with a chromophore are the main components that could interfere with the UV-detection of the target components. Caffeine is a potential interference component because it meets all the criteria. As caffeine is prohibited as an additive in e-liquids [3], the sample would not be conform anyhow.

Further information on the selectivity of the method was obtained when purchased e-liquid samples were investigated with both DAD and MS-detection as a proof of applicability (see 3.3).

3.2.2 Linearity of the calibration curve

The calibration curve is established from five calibration points in a concentration range mentioned in Table 6.4 by applying least square linear regression. An ordinary/unweighted linear regression model was chosen because of the narrow range. The linearity was confirmed using R^2 values, the quality coefficient (QC) and the Mandel fitting test. The R^2 and QC-values are summarized in Table 6.4 with all R^2 values being above 0.999 and the QC-values below 2.5%. The Mandel fitting test was not significant for the target components indicating that there is no significant difference between a linear and quadratic calibration model. In this case, the linear model is preferred [52].

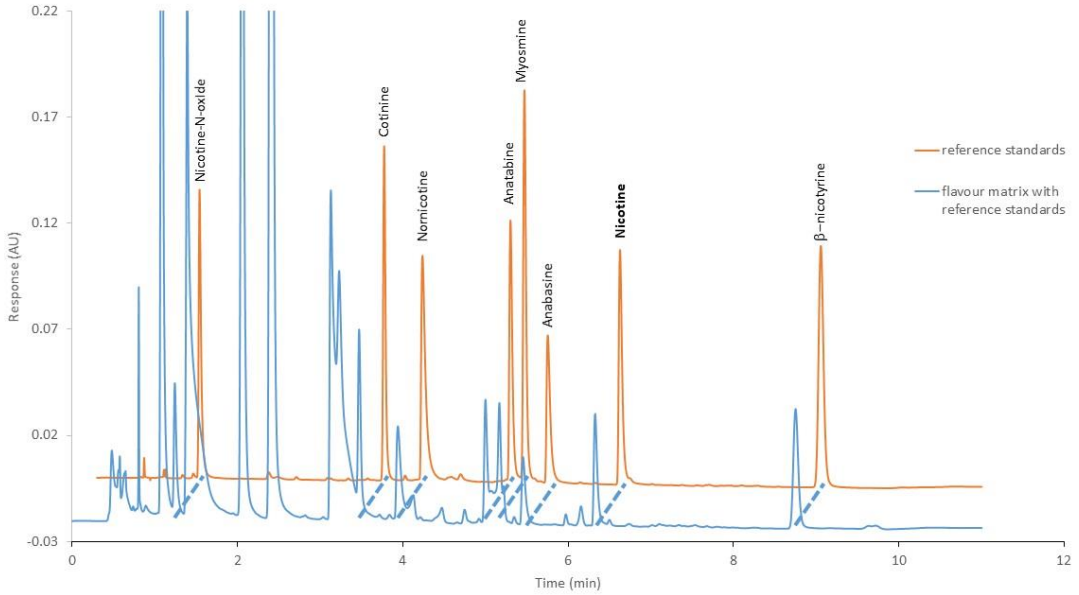


Figure 6.2: *Chromatogram of the target components obtained with the optimized UPLC-DAD method. (AU = absorbance units)*

3.2.3 Method linearity

The linearity of the results, demonstrated as the relationship between the measured concentration and the theoretical concentration, is linear with R^2-values above 0.9999 for all components (Table 6.5).

Table 6.4: The method (not instrumental) LOQ, LOD and validation of the linearity of the calibration curve for nicotine and its related impurities using R^2 - and QC-values obtained for all the calibration lines during method validation.

	R^2	QC %	LOD µg/ml	LOQ µg/ml
Anabasine	0.9996	2.02	0.59	2.23
Anatabine	0.9997	1.63	0.40	1.97
β–Nicotyrine	0.9997	1.10	0.31	20.16
Cotinine	1.0000	0.56	0.18	2.22
Myosmine	0.9999	1.02	0.07	2.29
Nicotine	0.9998	1.58	0.49	4.98
Nicotine-N-Oxide	0.9999	0.83	0.31	2.68
Nornicotine	0.9999	1.20	0.56	4.68

3.2.4 Trueness and Precision

The trueness is the closeness of agreement between the average value of a series of measurements and the true value, in this case the exact known concentration of the validation samples. It estimates the systematic error of an analytical method and is expressed as a relative bias at each concentration level. As shown in Table 6.5, the relative bias for all the components was well below 10% with a maximum relative bias of 6.98% for the highest level of myosmine. Consequently, the validation requirements are fulfilled.

The precision is the closeness of agreement between the values obtained from repeated measurements. It estimates the random error of the method and is expressed using relative standard deviation (RSD). For each concentration level, the repeatability was obtained from the variability of the triplicate measurements. The intermediate precision was investigated for the time-dependent variability of the method. The results are displayed in Table 6.5. The highest value was seen for β-nicotyrine with an intermediate precision of 7.90% which was considered acceptable, as also confirmed by the accuracy profiles.

Table 6.5: Results of the validation of the method linearity, trueness, precision and accuracy of the LC-DAD method for the quantification of nicotine and its minor alkaloid impurities (L = Level).

	R^2	Relative bias (%)			repeatability			intermediate reproducibility			β-expectance tolerance interval					
		L1	L2	L3	L1	L2	L3	L1	L2	L3	L1		L2		L3	
Anabasine	0.99996	4.85	1.56	5.26	3.90	1.93	1.57	5.41	5.00	4.11	[-9.54;	19.24]	[-12.59;	15.71]	[-6.07;	16.58]
Anatabine	0.99992	-6.23	-6.43	0.46	3.15	1.04	1.10	5.10	3.28	6.10	[-19.60;	7.15]	[-17.26;	4.40]	[-18.13;	19.05]
β–Nicotyrine	0.99997	-2.61	0.36	-0.04	7.55	2.87	1.27	7.90	3.77	3.66	[-19.88;	14.67]	[-11.18;	11.90]	[-18.06;	17.97]
Cotinine	1.00000	2.86	3.02	2.78	2.90	1.89	1.66	3.49	2.16	2.58	[-7.09;	12.81]	[-2.43;	8.46]	[-4.20;	9.76]
Myosmine	0.99984	-0.73	-1.00	6.98	3.13	1.21	3.23	1.57	0.96	1.61	[-15.83;	14.37]	[-4.35;	2.35]	[-3.53;	17.49]
Nicotine	0.99999	-0.19	-0.94	1.23	2.12	1.32	2.20	2.17	1.75	2.20	[-5.61;	5.23]	[-6.21;	4.33]	[-4.32;	6.77]
Nicotine-N-Oxide	0.99999	2.04	1.37	2.92	2.01	1.36	1.46	3.00	2.07	2.72	[-8.91;	12.99]	[-4.48;	7.22]	[-5.61;	11.45]
Nornicotine	0.99968	-5.70	-7.71	-3.56	3.14	4.00	0.65	4.06	4.00	3.76	[-15.84;	4.43]	[-15.94;	0.53]	[-16.44;	9.33]

3.2.5 Accuracy and LOQ

Based on the obtained trueness and precision of the method, the β-expectation tolerance intervals, representing the accuracy of the method, were calculated. Accuracy takes the total error associated with each measurement into account. The accuracy profiles of nicotine and its specified alkaloids are presented in Figure 6.3 and the β-expectation tolerance intervals are given in Table 6.5. The accuracy profiles show that the β-expectation tolerance intervals do not exceed the acceptance limits of ± 10% for nicotine and ± 20% for the specified impurities. Therefore, this method is considered suitable for the intended purpose.

The LOQ is also determined from the accuracy profiles and is defined by the concentration where the β-expectation tolerance interval crosses the acceptance limit. If the β-expectation tolerance intervals do not cross the acceptance limits, the lowest tested spiking level can be considered as the LOQ, as is the case in this study. The LOQ are given in Table 6.4. The LOQ of β-nicotyrine is the highest because accurate results were difficult to obtain in the lower concentration range although the relative response factor of β-nictoyrine was high compared to nicotine and the other impurities.

3.2.6 Limit of detection

The LOD was estimated as the concentration with a signal-to-noise ratio of at least three, as recommended by the International Council for Harmonisation (ICH) guidelines [22]. Samples with known decreasing concentrations were analysed to empirically determine the LOD. The LOD was determined as the concentration where the signal-to-noise ratio of the resulting peak was equal or higher than three. The obtained results for nicotine and its specified impurities are given in Table 6.4.

However, it is currently not possible to recommend a required sensitivity for the analytical methods intended for e-liquid analysis as there are no inhalation toxicity limits available for e-cigarettes. Further research is necessary to determine the minimum nicotine concentration in e-liquids that exerts a physiological response, in order to determine relevant limits of detection for nicotine and its impurities.

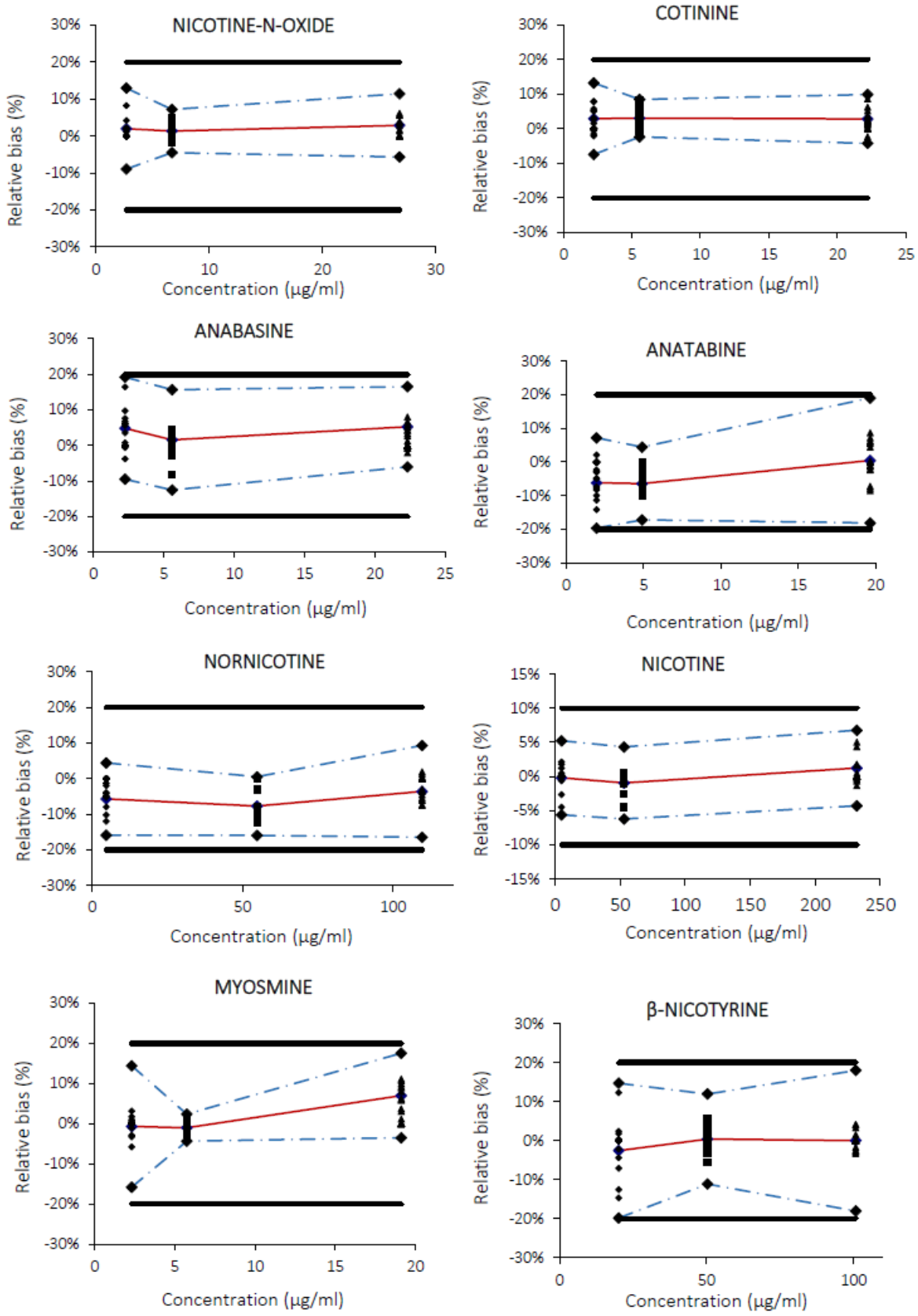


Figure 6.3: *Accuracy profiles obtained for the target components with β set at 95%. Legend: Relative bias (–), upper and lower β-expectation tolerance limits (– •), upper and lower acceptance limits set at 10% or 20% (–), relative back-calculated concentrations per spiking level (◆▲■).*

3.2.7 Recovery

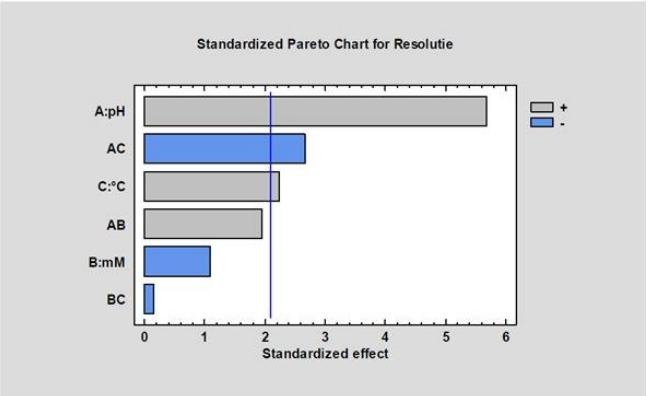
The method was validated using spiked 70/30 propylene glycol/glycerol matrix. To evaluate the method for other e-liquid matrix compositions, recoveries were determined for a 100% propylene glycol, 100% glycerol and 50/50 propylene glycol/glycerol matrix spiked at the intermediate concentration level in triplicate. The results are shown in Table 6.6. The obtained recoveries were all between 90% and 110%. Thus, the method is validated for all possible ratios of propylene glycol and glycerol in the e-liquid matrix.

Table 6.6: Recovery of the target component in matrices with different ratios of propylene glycol and glycerol in the matrix (analyzed in triplicate). Matrix 1= propylene glycol; matrix 2= 50:50 propyleneglycol/glycerol; matrix 3= glycerol

		Matrix 1		**Matrix 2**		**Matrix 3**	
	µg/ml	**average**	**SD**	**average**	**SD**	**average**	**SD**
Nicotine-N-Oxide	5	103.13	± 1.43	103.26	± 0.92	102.62	± 0.84
Cotinine	5	104.87	± 0.99	105.54	± 1.55	106.98	± 0.69
Nornicotine	50	94.73	± 2.37	93.37	± 2.40	91.41	± 0.58
Anatabine	5	101.29	± 1.15	100.21	± 0.91	92.36	± 2.02
Myosmine	5	100.69	± 0.24	99.02	± 0.45	100.35	± 1.49
Anabasine	5	97.15	± 4.18	90.70	± 4.37	92.95	± 7.98
Nicotine	50	100.17	± 0.38	99.66	± 0.65	96.39	± 1.78
β–Nicotyrine	50	103.87	± 1.32	103.56	± 0.37	102.02	± 2.34

3.2.8 Robustness

The robustness of the UHPLC-DAD method was investigated. As can be seen in Figure 6.4, only the pH and the column temperature had a significant effect on the resolution (p-values <0.05). The parameter which had the most influence on the results and thus should be controlled is the pH of the ammonium borate buffer (p-value <0.0001). This was also noticed during the method development where a small adjustment to the buffer pH resulted in a significant different peak separation. The column oven temperature had a small, but significant effect, though not as pronounced as the mobile phase buffer pH (p-value of 0.0364) since the resolution varies between 2.64 and 2.79. Small changes in the buffer concentration of the mobile phase do not have an apparent effect on the peak separation. The method can be considered as robust when the buffer pH of the mobile phase is controlled sufficiently. Other factors result only in small changes of the resolution.



Analysis of Variance for resolution

Source	Sum of Squares	Df	Mean Square	F-Ratio	P-Value
A:pH	14,6521	1	14,6521	32,21	0,0000
B:mM	0,544272	1	0,544272	1,20	0,2871
C:°C	2,2898	1	2,2898	5,03	0,0364
AB	1,74803	1	1,74803	3,84	0,0641
AC	3,22403	1	3,22403	7,09	0,0150
BC	0,00963333	1	0,00963333	0,02	0,8858
Total error	9,09921	20	0,454961		
Total (corr.)	31,5671	26			

Figure 6.4: *Pareto chart of the effects for the robustness study. The effect is represented by the resolution of the critical peak pair. The investigated factors were the pH (A) and molar concentration (B) of the ammonium borate buffer and the column temperature (C). Values with * are significant p <0.05.*

3.3 Proof of applicability

To proof the applicability of the UHPLC-DAD method, a small sample set of e-liquids was investigated. The samples were quantified using the validated UHPLC-DAD method and the results compared to those obtained using a screening LC-MS/MS method.

The results are summarized in Table 6.7. Out of the 10 samples investigated, 4 samples were labelled as zero liquids (INT 1, INT2, VS2, VS5) and thus should not contain nicotine. However, it was found that only 2 out of these 4 labelled “zero liquid” samples (INT1 and VS5) did not contain any nicotine nor impurities, whilst traces of nicotine were present in the other two (INT2, VS2). Screening with LC-MS/MS showed that only INT2 contained a nicotine-related impurity. Although nicotine-N-oxide was detected during screening in INT2, the concentrations were below the LOD of the UV-detection method as no other peaks were detected at the retention time of the impurity nicotine-N-oxide.

Table 6.7: Analysis of commercial e-liquid samples with the UHPLC-DAD quantification method and targeted LC-MS/MS screening method. INT = internet samples, VS = samples bought in specialized vapeshops. Concentration of the impurities are expressed in µg/g and nicotine concentrations in mg/ml.

	INT1[1]		INT2[1]		INT3		INT4		INT5	
	MS/MS	UV	MS/MS	UV	MS/MS	UV	MS/MS	UV	MS/MS	UV
COT	-	<LOD	-	<LOD	+	<LOD	+	13.13 ± 0.03	+	<LOQ
NNO	-	<LOD	+	<LOD	+	136.10 ± 4.73	+	405.23 ± 0.35	+	30.63 ± 0.48
NOR	-	<LOD	-	<LOD	+	<LOQ	+	<LOQ	+	9.65 ± 0.64
ANAB	-	<LOD	-	<LOD	+	<LOD	+	42.03 ± 1.90	+	<LOQ
ANAT	-	<LOD	-	<LOD	-	<LOD	+	38.15 ± 2.36	+	<LOQ
NIC	-	<LOD	+	1.78 ± 0.32	+	8.56 ± 0.00	+	19.80 ± 0.00	+	8.33 ± 0.01
BNIC	-	<LOD	-	<LOD	-	<LOD	+	<LOQ	-	<LOD
MYO	-	<LOD	-	<LOD	+	2.19 ± 0.37	+	29.05 ± 3.14	+	3.82 ± 0.30

Table 6.7 (continued): Analysis of commercial e-liquid samples with the UHPLC-DAD quantification method and targeted LC-MS/MS screening method. INT = internet samples, VS = samples bought in specialized vapeshops. Concentration of the impurities are expressed in µg/g and nicotine concentrations in mg/ml.

	VS1		VS2[1]		VS3		VS4		VS5[1]	
	MS/MS	UV	MS/MS	UV	MS/MS	UV	MS/MS	UV	MS/MS	UV
COT	+	2.99 ± 0.04	-	<LOD	+	<LOQ	-	<LOD	-	<LOD
NNO	+	9.92 ± 0.98	-	<LOD	+	21.41 ± 0.29	+	6.03 ± 2.40	-	<LOD
NOR	+	<LOQ	-	<LOD	+	<LOQ	+	6.90 ± 0.59	-	<LOD
ANAB	+	<LOQ	-	<LOD	-	<LOD	-	<LOD	-	<LOD
ANAT	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD
NIC	+	10.65 ± 0.03	+	1.08[2]	+	5.95 ± 0.02	+	2.99 ± 0.00	-	<LOD
BNIC	-	<LOD	-	<LOD	-	<LOD	-	<LOD	-	<LOD
MYO	+	3.36 ± 0.38	-	<LOD	+	<LOQ	+	<LOQ	-	<LOD

[1]*E-liquids containing 0 mg/ml according to the label*

[2]*No duplicate measurement available*

Nicotine-containing e-liquid samples were analysed as well. In general, all nicotine-containing samples contained the related-nicotine impurities (Table 6.7). Most of the impurities identified with the targeted MS/MS-method, were also found with the UHPLC-DAD method. The impurities were further quantified if the concentration allowed it (>LOQ). In sample INT3 cotinine and anabasine were found during the screening with LC-MS/MS, however similar to INT2 above, the concentrations were high enough to be detected during screening (>50 ppb), but below the LOD of the UV-method. Nevertheless, the results of the analysed e-liquid samples indicate that there still might be a quality issue regarding impurities content and the label conformity of nicotine.

In conclusion, the UHPLC-DAD method is suitable for the quantification of nicotine in e-liquids as the results of the nicotine analysis with UV-detection and targeted MS/MS screening are similar and the limiting factor of the UHPLC-DAD quantification is the sensitivity rather than selectivity. Impurities are analysed for different purposes and depending on the purpose, UV-detection could be acceptable or a MS-screening approach is preferred. For instance, when establishing impurities profiles, the sensitivity of MS-detection is important in order to include as many impurities as possible regardless of their concentrations. On the other hand, if the impurities are investigated for quality purposes, the conformity limits will determine the sensitivity of the adopted method. For example, previous investigation of nicotine impurities in e-liquids applied the impurities limits described in the Ph. Eur. monograph of nicotine for pharmaceuticals as conformity limits [33]. According to this monograph the limits for the specified nicotine impurities is 0.3% of the nicotine concentration. Thus, the minimum concentration the quantification method is required to determine (LOQ) is 9 µg/ml if the nicotine concentration is 3 mg/ml. This concentration is the lowest available nicotine concentration in commercial e-liquids hence, 9 µg/ml is the minimal LOQ of a method for quality control purposes when Ph.Eur limits are applied. Thus, with the LOQ of the UV-detection method all alkaloids are quantifiable (with the exception of β-nicotyrine) below this pharmacopoeial limit. Hence the sensitivity of the UV-detection is sufficient to detect e-liquids with nicotine impurities exceeding the Ph.Eur limits.

4 CONCLUSION

In this work, a simple dilute-and-shoot UHPLC-DAD method was developed for the simultaneous quantification of nicotine and its related impurities. The UHPLC-DAD method was successfully validated using accuracy profiles. The advantages of a UV-detection is that the method is more robust than LC-MS and produces results which are more accurate and precise without the need of an internal standard for quantification. Because of the complicated matrix and the presence of different flavour compounds in e-liquids, attention should be given to the selectivity and specificity of methods. The

analysis of the target components in the presence of a specific set of flavour components was therefore investigated. It was found that the interference and co-elution of flavouring components with the target components was limited for the 'flavoured' spiked validation samples.

To proof its applicability, the method was used to analyse zero-liquids and nicotine-containing e-liquids. The samples were also analysed by LC-MS/MS in order to qualitatively compare UV-detection to the standard method used for the analysis of nicotine impurities. Slightly different results were obtained between the MS- and UV-detection method, mainly due to the difference in sensitivity of the detection techniques, especially for the nicotine impurities. Nonetheless, the sensitivity of the UHPLC-DAD method is acceptable in the context of quality control of nicotine specified impurities, for which currently the Ph. Eur. limits are applied. Whereas for nicotine trace analyses of zero-liquids, the required sensitivity of the method depends on the purpose of the characterization (safety *versus* quality assessment). Considering safety assessment, the inhalation toxicity limits will be more important while for quality assessment the conventional limits, such as Ph.Eur. or United States Pharmacopoeia (USP) limits will be more relevant.

CHAPTER VII - IMPACT OF THE REVISED EUROPEAN TOBACCO PRODUCT DIRECTIVE ON THE QUALITY OF E-CIGARETTE REFILL LIQUIDS IN BELGIUM

Since the introduction of e-cigarettes, a lot of research has been performed to assess their safety and quality. From available literature on the chemical characterization of e-liquids (Chapter III), three main quality/safety issues were identified:

i) the presence of harmful flavourings such as diacetyl and acetylpropionyl (Chapter V),
ii) the discrepancy between the actual nicotine content and claimed nicotine concentration in an e-liquid (Chapter VI) and
iii) the presence of impurities of e-liquid ingredients including nicotine-related impurities (Chapter VI) and VOCs derived from flavourings.

During the course of this PhD project, the revised TPD, aimed at improving the quality and safety of e-cigarettes, came into force. In this chapter, we investigated the impact of the newly implemented criteria in the TPD on the quality of e-liquid refills available on the Belgian market. Hence, the methods developed in the previous Chapters V and VI were applied to investigate the quality of e-liquid samples acquired before (2013-2015), during (2016) and after (2017-2018) the implementation of the revised TPD.

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1 INTRODUCTION

Since its introduction, more than 10 years ago, the number of e-cigarette users is only increasing. Indeed, a study performed in the UK demonstrated that in 2018 almost 2/3 of tobacco smokers have tried the e-cigarette and 20% of them have continued to use them [53], [54]. To assure the quality and safety of these consumer products, the European Parliament approved in 2014 a revision of the Tobacco Products Directive 2014/40/EU (TPD) [3] that includes since then also a set of regulations concerning e-cigarettes. Since the TPD revision, nicotine-containing e-cigarettes are classified as tobacco products. In Belgium this means that they are no longer considered as a medicine, suspending their *de facto* ban that existed until then.

The revised TPD includes a set of more stringent measures concerning promotion and packaging/warning labels of e-cigarette products, limited maximum volumes for cartridges and e-

liquids. In addition, manufactures are required to notify the competent authorities before placing their products on the EU market. Moreover, stricter requirements are put forward for the ingredients present in the e-liquids, rendering their chemical characterization essential to be able to monitor compliance to the TPD and to control the quality of the available products on the market.

Our strategy to assess the quality of e-liquids is first of all based on the nicotine concentration accuracy. This is an indicator of good manufacturing practices during production of e-liquids. Nicotine in e-liquids and e-cigarette aerosols has been extensively investigated and studies performed prior to the implementation of the TPD, report a number of discrepancies between the actual nicotine concentration in the e-liquid and the labelled concentration [31], [32], [36], [40], [55], [56].

The nicotine concentration accuracy is, however, not enough to assure the quality of these products. Therefore, extra criteria need to be assessed, based on the three main requirements mentioned in the TPD concerning the e-liquid ingredients. These include 1) the use of high purity ingredients 2) prohibition of additives associated with energy and vitality and 3) the used ingredients may not pose any health risk in heated or unheated form.

For the first criterion this means that the impurities present in e-liquids need to be investigated. Aside from the ingredients intentionally added to e-liquids, the main focus of e-cigarette research has so far been the analysis of harmful and potentially harmful constituents. These include: (i) thermal degradation components formed during heating such as formaldehyde, acetaldehyde and acrolein, (ii) leachables from the e-cigarette device such as metals, but also (iii) impurities related to main e-liquid ingredients such as nicotine-related impurities, tobacco-specific nitrosamines (TSNAs), ethylene glycol (EG), diethylene glycol (DEG) and volatile organic solvents (VOCs) [20], [28], [57]–[64]. The latter group is thus indirectly mentioned in the TPD. In this study, the e-liquids are investigated for the presence of nicotine-related impurities and VOCs.

The second ingredient-related requirement is the prohibition of certain additives. E-cigarettes, containing lifestyle medicines (e.g. phosphodiesterase-5 inhibitors and slimming products) as well as drugs (e.g. MDMA) have been encountered [65], [66]. In fact, a whole new niche of e-liquids has emerged in which vitamins and supplements such as vitamin B12 and melatonin (VitaCig, InhaleHealth) are added in order to achieve potential health benefits via vaping. The additives evaluated in this study are the stimulants caffeine and taurine.

The third criterion does not exclude explicitly a specific ingredient or component, but includes all possible harmful ingredients, such as the added flavourings. E-liquids are available in more than 7000 flavours [67], which could include potential dangerous inhalation toxicants (e.g. diacetyl-

acetylpropionyl) [13], [68], [69]. Furthermore, it stands to reason that all these different flavours, represent a big challenge for regulating authorities in order to assess the health risk related to the inhalation of these components [70]. In our study, we assessed the presence of diacetyl and acetylpropionyl in the flavoured e-liquids.

Regardless of the implementation of the TPD, there is still no clear overview or systematic inspection of the quality of the different e-liquids available on the market. By investigating the chemical composition of e-liquids, according to the abovementioned strategy, a comparison of the quality of the different e-liquids is possible. The nicotine concentration accuracy was investigated in samples acquired before (2013-2015), during (2016) and after (2017-2018) the implementation of the revised TPD. Additionally, nicotine stability in e-liquid matrices was investigated for up to 9 months. The other components (nicotine-related impurities, VOCs, the additives caffeine and taurine and the flavours diacetyl and acetylpropionyl) were only investigated in samples acquired during and after the implementation of the revised TPD and will be compared with data from previous studies performed before the implementation of the TPD.

2 MATERIALS AND METHODS

2.1 Sample collection

A total of 246 e-liquids were analysed; 159 originating from Belgian vapeshops, 23 samples were bought online (prior to the implementation of the TPD), 3 Do-It-Yourself (DIY) samples and 61 samples originated from the Federal Agency for Medicines and Health Care Products (FAMHP). The latter samples were either obtained upon inspections of different vaping shops in Belgium or were seized postal packages ordered by individuals through the internet. All samples were stored at 4°C and protected from light. The target components were not analysed for all acquired samples. More details about which samples were investigated for which target component is given in Table 7.1.

The selection of the e-liquids was based on different criteria depending on the target components to be investigated. For nicotine all 246 samples were analysed. These include samples from different periods; before, during and after TPD implementation. The first series of samples was provided to us in the period of 2013-2015 by the FAMHP. These samples were either obtained upon inspections of different vaping shops in Belgium or were seized postal packages ordered by individuals through the internet. During this period Belgian law considered e-cigarettes and related products containing nicotine as medicinal products, which was de facto a ban on nicotine-containing e-cigarettes. This is the reason why the majority of these samples include zero-liquids. The second series of samples was collected in the period during the implementation of the Tobacco related Products Directive

2014/40/EU (TPD) between October 2016 and February 2017. At that time there were no regulations for e-cigarettes in Belgium because of juridical issues, leading to a grey zone. The third series of samples was collected after the final implementation of the TPD in the Belgian national law. The collecting period was between June 2017 and June 2018.

Table 7.1: The investigated sample set and number of samples for each component. The method for each component is given with the respective LOD and LOQ of the method used for e-liquid analysis. (QQQ = triple quad, IT = ion tap, HS = headspace, DAD = diode array detection, SIM = single ion monitoring)

	SAMPLE SET	NUMBER SAMPLES	SCREENING METHOD	LOD (µg/g)	QUANTIFICATION METHOD	LOQ (µg/g)
Nicotine	2013-2018	246	LC-MS/MS (QQQ)	0.1	UHPLC-DAD	5
Nicotine impurities						
anatabine	2016-2018	128	LC-MS/MS (QQQ)	0.1	UHPLC-DAD	2
anabasine				0.1		2
cotinine				0.1		2
nornicotine				0.1		5
myosmine				0.1		2
nicotine-N-oxide				0.1		2
Additives						
taurine	2017-2018	112	LC-MSn (IT)	100		
caffeine			GC-MS	1	UHPLC-DAD	5
Residual ingredients						
naphtalene	2016-2018	128	HS-GC-MS (SCAN)	0.2	HS-GC-MS (SIM)	1
xylene				0.2		0.5
ethylbenzene				0.05		0.07
hexane				2.5		2.5
isopropanol				1.5		1.5
Flavourings						
diacetyl	sweet-browns flavoured samples 2017-2018	40	HS-GC-MS (SCAN)	3	HS-GC-MS (SIM)	5
acetylpropionyl				8		5

Nicotine specified impurities were only investigated in the second sample set which included a large part of tobacco-flavoured e-liquids and zero-liquids. The second sample set is the only series which include samples from vapeshops, from the internet, from individuals selling DIY e-liquid samples and samples provided by the FAMHP. The residual solvents were investigated in the second and third sample set. For diacetyl and acetylpropionyl we focused only on sweet flavoured e-liquids from the third sample set. Sweet-flavoured e-liquids include fruit, dairy (butter, cheesecake, yogurt), brown (caramel, vanilla coffee, chocolate) and cocktail (pina colada) flavourings similar to the sample selection of Allen et al. 2016 [13]. The additives caffeine and taurine were only screened in the third

sample set bought after the implementation of the TPD (90 samples). In this third sampling set different flavours were included to have a representative set of the flavours on the market and specifically more e-liquids with caffeine/energy-associated flavours were included.

2.2 Standards and reagents

The standards of nicotine, cotinine, anabasine and myosmine and boric acid were purchased from Sigma Aldrich (St. Louis, USA). Standards nornicotine and β-nicotyrine were bought from Toronto Research Chemicals Inc. (Toronto, USA). Nicotine-N-oxide and anatabine came from Cayman Chemical (Michigan, USA). The reference standards diacetyl (2,3-butanedione) and acetylpropionyl (2,3-pentanedione) were purchased from Sigma-Aldrich (St. Louis, USA) and 1-butanol, used as an internal standard, was bought from Merck (Darmstadt, Germany). All standards had a purity grade of more than 97%. Solvents acentronitrile, methanol and acetone were HPLC-grade and purchased from Biosolve (Valkenswaard, The Netherlands). Concentrated ammonia 28-30%, analytical grade dimethylsulfoxide (DMSO) and sodium chloride were obtained from Merck (Darmstadt, Duitsland). E-liquid components propylene glycol, glycerol and 1,3-propanediol were purchased from Merck (Schuchardt, Germany). Water was obtained using a milliQ-Gradient A10 system (Millipore, Billerica, USA).

2.3 Nicotine and nicotine-related impurities

Nicotine and the **nicotine-related impurities** were first screened with LC-MS/MS, followed by a quantification with UHPLC-DAD. The used methodology was previously developed by our research group and is described in the previous Chapter 6 including the validation of these analytical methods for e-liquids. The same method was used to investigate the stability of nicotine in different e-liquid matrices.

2.4 Volatile components: residual solvents, diacetyl and acetylpropionyl

For the analysis of volatile components we opted for analysis through headspace-GC-MS. The collected e-liquid samples were simultaneously screened for the presence of volatile components that are classified as **residual solvents** by the ICH and the potential toxic flavourings **diacetyl and acetylpropionyl**. The method was based on the method previously describes in Chapter V with some adaptions. The screening was performed on an Agilent 6890 N gas chromatograph coupled to an Agilent 5973N triple quadrupole mass spectrometer and equipped with a G188A static headspace sampler (Agilent Technologies, Palo Alto, USA). The samples were first diluted by dissolving 1 g of e-liquid sample in 10 ml water. The incubation temperature of the headspace oven was maintained at 85°C with an equilibration time of 15min. The injector port was kept at 160°C, in split injection mode

(split ratio 15:1), while the temperatures of the headspace loop and the transfer line were maintained at 100 and 120°C, respectively. The components were separated on a VF-5 ms (5% phenyl-95% methylpolysiloxane) capillary column of 60m with Ø 0.25mm and film thickness of 0.2 5µm and an integrated guard column of 10 m (#CP9013, Factor four, Agilent, California, USA). Helium carrier gas was used at a constant flow of 1.0 ml/min. The initial oven temperature of 45°C was held for 10 minutes. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Temperatures of the ion source, the quadrupole, and the interface were set at 230, 150 and 280°C, respectively. The chromatograms of the screening were obtained in full scan mode from 25 to 400 m/z. The quantification was carried out with the same chromatographic HS-GC-MS method in SIM mode (Table 7.2).

Table 7.2: Single ion monitoring (SIM) parameters for HS-GC-MS analysis of the volatile components. (RT = retention time, m/z = mass-to-charge ratio)

Compounds	RT (min)	qualifier (m/z)	quantifier (m/z)
diacetyl	6.1	86	43
acetylpropionyl	8.9	57	43
naphtalene	16.7	129	128
xylene	13.7	106	91
ethylbenzene	13.6	106	91
hexane	6.25	57	86
isopropanol	5.2	45	43

2.5 Caffeine

Caffeine was first screened in e-liquids samples with GC-MS. The samples were diluted by dissolving 1 g of e-liquid samples in 10 ml of methanol. A volume of 1 µL of the solution was injected in split mode (50:1). The initial temperature of the GC oven was 80°C. After a hold of 2 min, the temperature gradient was started by augmenting the temperature to 280°C by 15°C/min. This makes the total runtime of the analysis 17 min. The He-gas flow was set at 1 ml/min constant flow and the screened masses were between 25 m/z and 600 m/z. Positive samples were further quantified by UHPLC-DAD. The samples were first diluted by dissolving approximately 1 g of e-liquid in 10 ml water. The analyses were conducted on an Acquity UPLC™ system (Waters, Milford, USA) using DAD-detection with a Waters Acquity BEH RP18 2.1 mm × 100 mm, 1.7 µm column and a Van Guard BEH pre-column (2.1 mm × 100 mm, 1.7 µm) to prolong the lifespan of the column. The gradient consisted of 0.1% formic acid in water as mobile phase A and acetonitrile as mobile phase B. The gradient started at 95% aqueous phase and 5% organic phase. After 6 min the gradient changed to 50% organic phase, which was held for 2 min, followed by the re-equilibration of the gradient to 95% aqueous phase. The

gradient had a flow of 0.4 ml/min. The injected volume was 10 µl in full loop mode for high reproducible results. Acetonitrile was used as the strong needle wash solvent and 95% water and 5% acetonitrile as the weak needle wash solvent. Sample temperature was set at 10°C and the column temperature at 30 °C. The wavelength used for the quantification was 273 nm.

2.6 Taurine

Taurine was screened by employing non-targeted screening using LC-MS/MS. Prior to injection, 1 g of the e-liquid samples were first diluted into 10 ml water. Next, 2 µl of these diluted samples were injected on to a Dionex UltiMate 3000 Rapid Separation LC system (Thermo Scientific, Sunnyvale, CA, USA) coupled to an amaZon™ speed ETD mass spectrometer (Bruker Daltonics, Bremen, Germany). The MS instrument system was calibrated using the manufacturer's calibration mixture, and the mass accuracy was determined to be <0.1 Da during the period of analysis. The chromatographic separation was performed at 40 °C on an Acquity UPLC HSS T3 Column (100 mm×2.1 mm, 1.7 µm) (Waters, Milford, MA, USA) with a mobile phase consisting of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The optimized elution method, with a constant flow rate of 0.3 mL/min, employed an isocratic step of 100% A for 1 minute, followed by a linear gradient to 95% A for 1.5 min, which was sufficient for the elution of the desired compound. However, an additional on-line cleaning step, consisting of a linear gradient to 10% A at 3 minutes, followed by an isocratic elution of 2 minutes with 100% B and a 2 minutes recalibration with 100% A (total run time 7 minutes) was necessary. The mass spectrometer settings were similar to what has been previously described in [71] however small adjustments were made to the mass range (100-300 m/z) and the smart parameter setting (m/z 200). The MS and MS/MS queries were performed using Compass® Data Analysis 4.2 (Bruker Daltonics, Bremen, Germany) software. A 0.2 Da precursor tolerance for MS spectra and a 0.2 Da fragment tolerance for MS/MS spectra were allowed.

2.7 Nicotine stability in e-liquid matrices

A stability study was set up to assess the influence of matrix type and light exposure on the stability of nicotine in e-liquids. Hereto, a subset of 18 self-made e-liquid samples were spiked with a nicotine standard to obtain 3 mg/g and 6 mg/g preparations. Four different e-liquid matrices were included: 100% propylene glycol – 100% glycerol and 50/50 propylene glycol/glycerol and 100% 1,3-propandiol. The samples were stored in polypropylene falcons for up to 9 months at 25 ± 2°C and 60 ± 5% relative humidity, in agreement with the long term climate type II conditions in the ICH guidelines [72]. Monitoring of the climate conditions was done using a Libero THi1 V3.24 datalogger. Additionally, in order to investigate the influence of light exposure, duplicates of all samples were prepared of which one was kept in the dark and the other not (natural light exposure through sunlight and fluorescent

indoor lighting). Nicotine concentrations and its related impurities were determined after preparation of the sample and 1, 3, 6 and 9 months after storage.

2.8 Statistics

The conformity of the samples between groups (vapeshop, internet) and before, during and after the implementation of the TPD were performed by using the Pearson Chi square test. Correlations were determined by using Pearson's correlation coefficient. All comparisons were two-tailed, and a p value of <0.05 was considered statistically significant.

3 RESULTS

3.1 Nicotine concentration label accuracy

Nicotine was analysed in so-called "zero-liquids" collected between 2013 and 2018. Zero-liquids are conform the TPD if the measured nicotine concentrations are below the detection limit (0.5 µg/ml). As can be seen from Figure 7.1, 47% of the claimed zero-liquids collected prior to the TPD implementation contained nicotine either in traces or in higher amounts [<LOQ – 4.2 mg/g] (Table 7.3). The zero-liquids collected in 2016 i.e. during TPD implementation, demonstrate a high % of non-conformity. More specifically, 50% of the internet zero-liquids contained nicotine compared to 38% of the zero-liquids obtained from the vapeshops. This might indicate that the quality of internet-bought samples are of lesser quality than the e-liquids from vapeshops. However, the majority of the zero-liquids in the sample set were tobacco flavoured (30%) and therefore a certain bias towards nicotine traces related to the tobacco flavouring extracts is present. The third set of samples, collected post TPD implementation and acquired from vapeshop inspections by the national health authorities in 2017 and 2018, demonstrate a higher level of conformity ($p < 0.05$) since only 4 zero-liquids were found to contain nicotine [<LOQ] on a total of 35 samples (11%).

Table 7.3: Nicotine concentration of positive tested zero-liquids.

CODE	year	source	nicotine concentration (mg/g)
15-1	2015	vapeshop	<LOQ
15-13	2015	vapeshop	<LOQ
15-14	2015	vapeshop	<LOQ
15-25	2015	vapeshop	<LOQ
15-26	2015	vapeshop	0.05
15-27	2015	vapeshop	<LOQ
15-28	2015	vapeshop	1.90
15-29	2015	vapeshop	2.60
15-30	2015	vapeshop	4.20
15-31	2015	vapeshop	3.30
15-32	2015	vapeshop	2.90
15-33	2015	vapeshop	2.70
15-34	2015	vapeshop	2.60
15-39	2015	vapeshop	<LOQ
15-40	2015	vapeshop	<LOQ
15-41	2015	vapeshop	<LOQ
15-42	2015	vapeshop	0.03
15-44	2015	vapeshop	0.03
15-45	2015	vapeshop	0.01
15-46	2015	vapeshop	0.02
15-48	2015	vapeshop	0.04
15-49	2015	vapeshop	<LOQ
15-50	2015	vapeshop	1.04
15-51	2015	vapeshop	0.04
15-52	2015	vapeshop	0.09
15-53	2015	vapeshop	0.09
15-54	2015	vapeshop	0.04
AS6	2016	vapeshop	0.01
VS7	2016	vapeshop	0.01
VS11	2016	vapeshop	0.02
INT7	2016	internet	0.03
INT10	2016	internet	0.08
INT11	2016	internet	0.09
INT13	2016	internet	1.22
INT14	2016	internet	1.78
INT15	2016	internet	<LOQ
INT17	2016	internet	<LOQ
INT20	2016	internet	<LOQ
8003-17-0066A	2017	vapeshop	<LOQ
1301-18-0182E	2018	vapeshop	<LOQ
1202-18-0227	2018	vapeshop	<LOQ
1301-18-0179E	2018	vapeshop	<LOQ

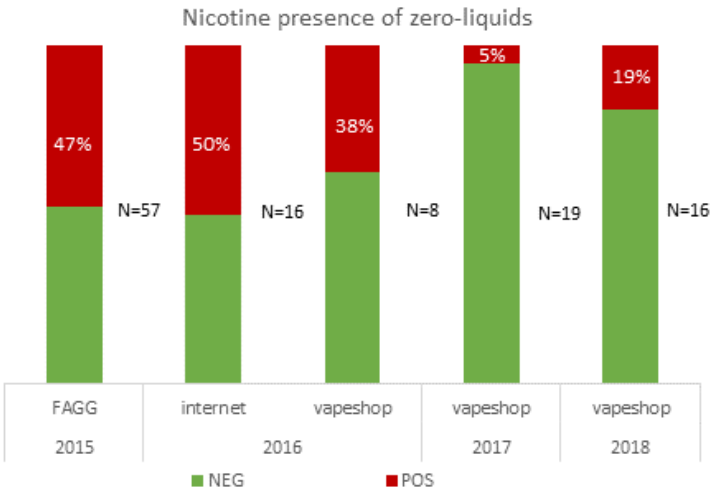

Figure 7.1: *Overview of the chemical characterization of the investigated e-liquid samples. Conformity of the tested zero-liquids (no nicotine present (NEG), nicotine present (POS)).*

Next, the conformity of nicotine-containing e-liquids was investigated by comparing the labelled *versus* the measured nicotine concentration. Currently, there are no criteria in the TPD that provide information with respect to the allowed deviation between the claimed and the actually nicotine concentration. For this reason, non-legally binding guidelines have been developed by the industry itself such as those of the American E-liquid Manufacturing Standards Association (AEMSA) and the Association Française de Normalisation (ANFOR). The AEMSA handles a tolerance level of +/-10% nicotine content in a final product, whereas this is only +/- 5% in the ANFOR guidelines [73], [74]. In our study, samples have been divided into three groups based on the outcome of the nicotine analysis.

The first category contains samples that deviate less than 10% from the claimed nicotine concentration. These samples are regarded as conform since the error of the analytical method (10%) is taken into account. The second category is the 'grey zone', containing samples with an actual nicotine concentration that deviates between 10% and 20% of the labelled concentration. Although the nicotine concentration is significantly different from what is claimed, it is not sure whether this also has an impact on the actual nicotine level present in the aerosols that will be inhaled. Indeed, to estimate the actual nicotine exposure, other factors such as the type of e-cigarette device and the user's topography are also important parameters to take into consideration. The third category contains samples with measured nicotine concentrations that deviate more than 20% of the claimed nicotine concentration. The limit of 20% is often regarded as not conform in other studies.

The results of the samples collected in 2016 show that 15% of the investigated samples have an actual nicotine concentration that deviates more than 20% from the labelled concentration whilst in the 2017-2018 sample set this was only 7% (Figure 7.2). The high number of non-conformity in 2016 is mainly due to the DIY- and internet samples ($p < 0.05$). For the samples coming from the vapeshops, no significant trend change could be observed from 2016 to 2018. Interestingly, the non-conform samples acquired from the vapeshops, contained less nicotine than labelled, whereas the non-conform DIY samples and the samples acquired from the internet contained a higher nicotine concentration than claimed (Table 7.4).

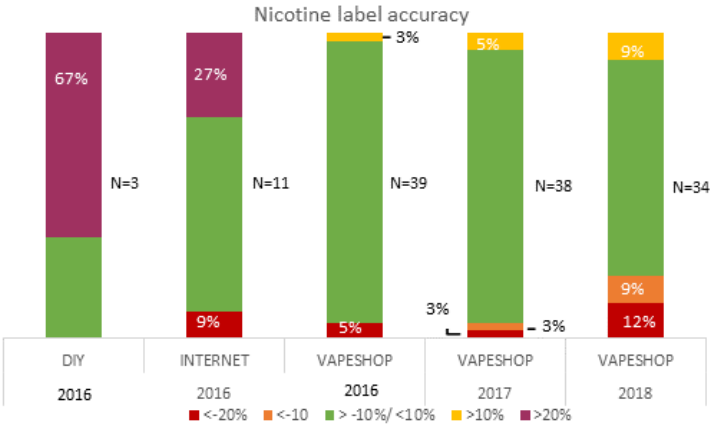

Figure 7.2: *Label accuracy of the nicotine concentration. The first category (green) contains samples that deviate less than 10% from the claimed nicotine concentration. These samples are regarded as conform as the error of the analytical method (10%) is taken into account. The second category (orange) is the grey zone that contains samples with a nicotine concentration deviating between 10% and 20% from the labelled concentration. The third category (red) contains samples with measured nicotine concentrations deviating more than 20% of the claimed nicotine concentration. (DIY = Do-It-Yourself e-liquids)*

Table 7.4: Label accuracy: Nicotine concentration(mg/ml) of e-liquid samples with an absolute deviation of more than 10%. (DIY = Do-It-Yourself e-liquids)

CODE	Source	Labelled concentration (mg/ml)	Year	Measured concentration (mg/ml)	%	% deviation
INT23	internet	6	2016	19.80	329.94%	>20%
DIY2	DIY	3	2016	5.79	192.93%	>20%
INT21	internet	6	2016	10.73	178.91%	>20%
DIY1	DIY	3	2016	5.28	176.05%	>20%
INT22	internet	6	2016	10.05	167.50%	>20%
AS8	vapeshop	6	2016	6.67	111.15%	>10%
AS9	vapeshop	12	2016	9.15	76.23%	<-20%
FAGG4	customs	8	2016	5.99	74.82%	<-20%
AS7	vapeshop	3	2016	1.78	59.21%	<-20%
2005-17-004	vapeshop	12	2017	14.23	118.55%	>10%
177163	vapeshop	12	2017	13.82	115.17%	>10%
2005-17-002	vapeshop	12	2017	10.71	89.27%	<-10%
2001-17-0010	vapeshop	12	2017	9.34	77.82%	<-20%
1301-18-0201E	vapeshop	12	2018	14.24	118.64%	>10%
1202-18-0221	vapeshop	6	2018	6.77	112.88%	>10%
1301-18-0190E	vapeshop	6	2018	6.76	112.74%	>10%
1202-18-0234	vapeshop	6	2018	5.40	89.95%	<-10%
1301-18-0181E	vapeshop	6	2018	5.27	87.82%	<-10%
1202-18-0238	vapeshop	3	2018	2.45	81.73%	<-10%
1202-18-0242	vapeshop	6	2018	4.43	73.77%	<-20%
1301-18-0186E	vapeshop	3	2018	1.78	59.37%	<-20%
1202-18-0229	vapeshop	3	2018	1.63	54.47%	<-20%
1202-18-0230	vapeshop	6	2018	2.80	46.72%	<-20%

3.2 Impurities: Nicotine-related impurities

Analysis of the nicotine specified impurities, present in the 2nd (2016) and 3rd (2017-2018) sample set demonstrate that the most abundant impurities present are nicotine-N-oxide and myosmine (Figure 7.3). Also cotinine, nornicotine, anatabine and anabasine were found in some nicotine-containing e-liquids. The only impurity that could not be detected was β-nicotyrine. In Table 7.5 the measured concentration for each alkaloid impurity is given in detail. However, not all positive identified samples could be quantified, as some of the impurities were present below the LOQ of the method or because of interference with other e-liquid components. The concentrations of the impurities were highly variable. The higher the nicotine concentration, the higher the concentration of impurities

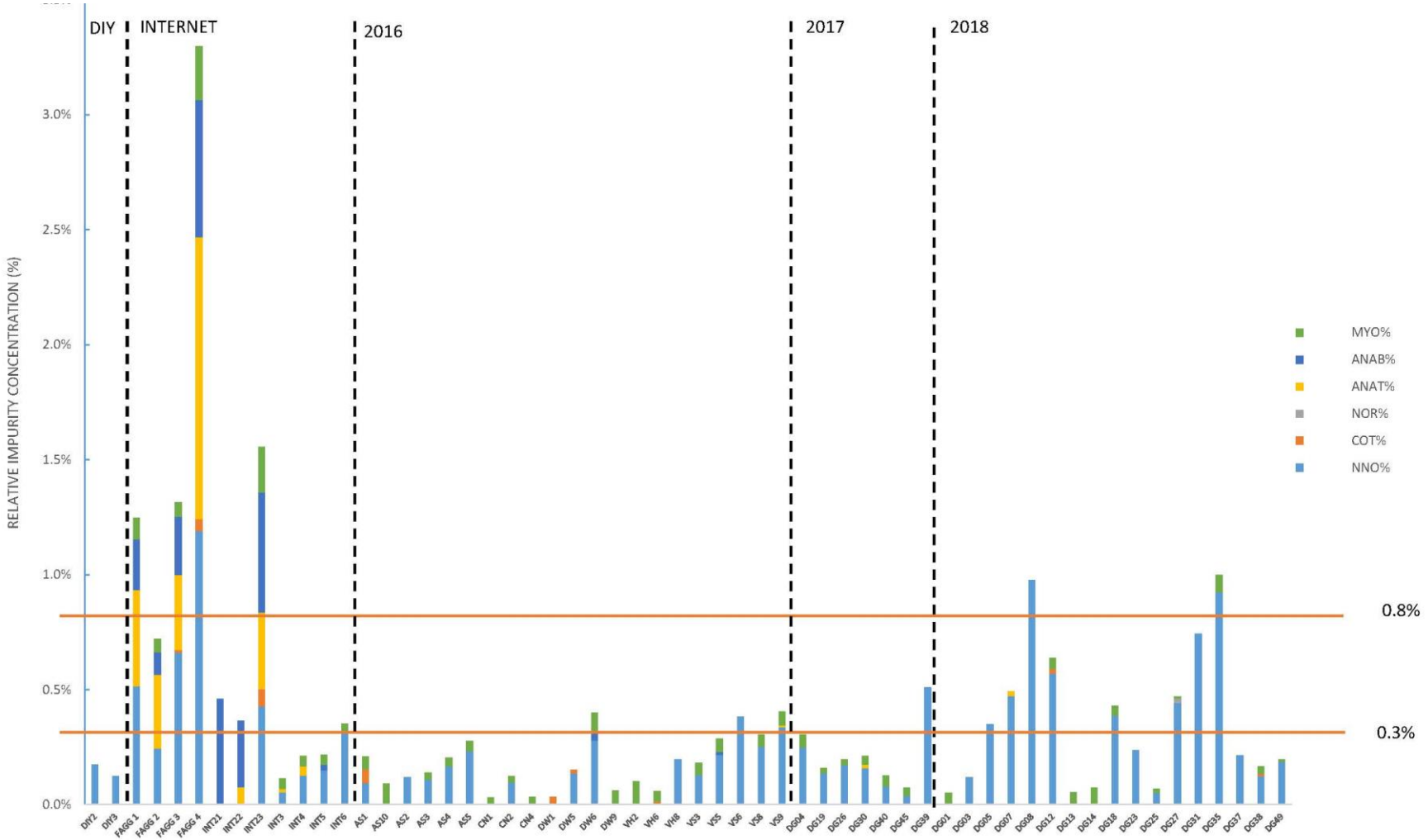

***Figure 7.3**: Presence of nicotine-related impurities in the investigated e-liquid samples. Nicotine impurities content (specified impurity (mg)/ nicotine (mg) %) of e-liquids, with individual specified impurity limit of 0.3% and total specified impurities limit of 0.8%. Samples exceeding the Ph.Eur. threshold are not conform (Myosmine (MYO), anabasine (ANAB), anatabine (ANAT), nornicotine (NOR), cotinine (COT), nicotine-N-oxide (NNO)).*

Table 7.5: Concentration of the nicotine-related impurities (IF: screened positive, interference in UV, (COT = cotinine, ANAB = anabasine,ANAT = anatabine, NOR = nornicotine, NNO = nicotine-N-oxide, MYO = myosine)

CODE	Year	Nicotine concentration (mg/ml)	NNO (µg/ml)	COT (µg/ml)	NOR (µg/ml)	ANAT (µg/ml)	MYO (µg/ml)	ANAB (µg/ml)
AS2	2016	2.99	3.56		<LOQ		<LOQ	
DIY2	2016	5.79	10.12				<LOQ	
VS6	2016	2.82	10.78				<LOQ	
AS1	2016	5.43	5.07	3.23	<LOQ		3.08	
AS4	2016	5.93	9.87		<LOQ		2.19	
CN4	2016	6.06	IF				2.10	
DIY3	2016	5.66	7.056				<LOQ	
INT21	2016	10.73	IF				<LOQ	49.36
INT22	2016	10.05	IF			7.68		29.08
INT23	2016	19.80	84.69	15.02		65.64	39.15	103.32
VH2	2016	5.90	IF				5.99	
VH8	2016	5.95	11.71	<LOQ			<LOQ	
AS10	2016	8.03	IF				7.40	
FAGG 4	2016	5.99	71.23	3.06		73.47	14.00	35.66
DW1	2016	9.12	IF	3.15			<LOQ	
INT3	2016	8.31	4.38		<LOQ	1.39	3.64	
INT4	2016	8.86	11.12	<LOQ	<LOQ	3.63	3.91	
INT5	2016	8.33	12.43	<LOQ	<LOQ	<LOQ	3.66	2.07
INT6	2016	8.56	26.55			<LOQ	3.58	
VS3	2016	8.90	11.49				4.67	<LOQ
DW6	2016	10.14	28.25			<LOQ	8.90	3.36
AS5	2016	11.08	25.73		<LOQ		4.91	
CN1	2016	11.10	IF				3.48	
CN2	2016	10.93	10.67				3.01	
FAGG 3	2016	11.69	77.11	1.44		38.10	7.36	29.65
VH6	2016	10.60	IF	1.49			4.66	
VS5	2016	10.65	23.20		<LOQ	<LOQ	6.11	1.1975
DW5	2016	11.96	16.44	1.57			<LOQ	
DW9	2016	17.18	IF				10.50	
FAGG 1	2016	18.00	92.95			75.26	16.64	39.50
FAGG 2	2016	17.71	43.47			56.62	10.15	17.49
VS8	2016	17.65	44.57				9.05	
VS9	2016	18.29	61.84			1.12	11.17	
AS3	2016	18.74	20.41		<LOQ		5.84	
DG26	2017	6.06	10.35				1.55	
DG30	2017	6.21	9.88			0.99	2.24	<LOQ
DG39	2017	6.01	30.58				IF	
DG04	2017	9.34	23.27				5.13	
DG19	2017	10.71	14.51				2.48	
DG40	2017	14.23	11.06				7.00	<LOQ

DG45	2017	12.32	4.88				4.33
DG03	2018	1.63	1.93				IF
DG07	2018	2.83	13.37			0.56	<LOQ
DG37	2018	3.27	7.02				IF
DG01	2018	2.80	IF				1.47
DG05	2018	6.06	21.16				
DG14	2018	6.43	IF				4.81
DG18	2018	6.16	23.84				2.67
DG25	2018	6.07	3.18	<LOQ			1.05
DG31	2018	6.76	50.33				
DG35	2018	5.27	48.73				3.85
DG38	2018	4.43	5.46	0.42			1.46
DG08	2018	11.88	116.01				
DG12	2018	11.48	65.38	2.52			5.41
DG13	2018	12.59	IF				6.79
DG23	2018	12.09	28.61				
DG49	2018	14.24	26.57	<LOQ		<LOQ	1.37
DG27	2018	18.17	80.53	<LOQ	3.36		1.57

The samples from 2016 contained more impurities and in higher concentrations compared to samples from 2017 and 2018. Especially the samples acquired from the internet shops contained more impurities compared to samples retrieved from vapeshops. Nicotine-N-oxide, which was found in all nicotine containing samples, was present in a concentration range of 3.55 – 345.25 µg/ml (median: 16.44 µg/ml). The other impurities were present in lower concentrations: 1.44 – 15.02 µg/ml (median: 3.06 µg/ml) and 2.10 – 39.15 µg/ml (median: 5.84 µg/ml) for cotinine and myosmine, respectively. Nornicotine was present in concentrations below the quantification threshold.

The other alkaloid impurities anatabine and anabasine varied highly between 1.12 – 75.26 µg/ml (median: 3.63 µg/ml) and 1.20 - 65.50 µg/ml (median: 29.37 µg/ml), respectively, depending on the brand. Until now the limits used for comparison of the nicotine alkaloids are those from the European Pharmacopoeia (Ph. Eur.). These limits are established for quality control purposes of nicotine used as an active pharmaceutical ingredient. These can be regarded as the minimal purity requirements for nicotine. The limits set for each specific impurity may not exceed the threshold of 0.3% relative to nicotine and a total relative concentration threshold of 0.8% is allowed. In Figure 7.3, the relative concentration of the quantified impurities is given for each sample.

The limit of 0.3% was exceeded for 20 e-liquids samples. In 16 of these samples, nicotine-N-oxide was the responsible alkaloid impurity, followed by anatabine and anabasine in 6 and 3 of the 20 samples, respectively. The other detected impurities were all present in relative concentrations below the Ph.

Eur. limit. The limit for the total relative concentration of impurities was exceeded by 6 e-liquid samples that also exceeded the 0.3% limit. The samples from 2016 contained more impurities and in higher concentrations as compared to samples from 2017 and 2018. Especially the samples acquired from the internet contained more impurities compared to the samples purchased in vapeshops.

3.3 Impurities: Residual solvents

The presence of **residual solvents** was investigated in 128 samples (2016-2018). Ethylbenzene, xylene, naphthalene, hexane and isopropanol were identified and confirmed in a total of 13 e-liquid samples using the HS-GC-MS screening. The results are summarized in Table 7.6. Napthalene, xylene and ethylbenzene were present in quantities lower than 0.5 µg/g. Hexane and isopropanol, regarded of lower risk to human health, were present in concentrations between 7.69 – 22.49 µg/g and 0.13 – 66.72 mg/g, respectively.

Table 7.6: Concentration (µg/g) of the residual solvents identified and quantified in e-liquid samples.

	naphtalene	xylene	ethylbenzene	hexane	isopropanol
VS8	<LOQ				
INT1				15.04	
INT10		<LOQ	0.17		
INT15			0.17		
INT17		<LOQ	0.29		
INT18			0.14		130.30
1206-17-004				22.49	
8001-17-005				7.69	5100.90
8003-17-0066A				10.32	
2001-17-0007				10.76	
1202-18-0239					130.30
1301-18-0186E					2030.93
VH4					66719.63

3.4 Additives: Caffeine and Taurine

The additives caffeine and taurine were screened in 112 samples bought after the implementation of the TPD. Taurine was not present in any of these samples. Caffeine, on the other hand, was identified in 12 of the e-liquid samples tested (Figure 7.4) with more positive samples in 2017 compared to 2018. The concentration varied from <LOQ (1 µg/ml) to 29 µg/ml (Table 7.7).

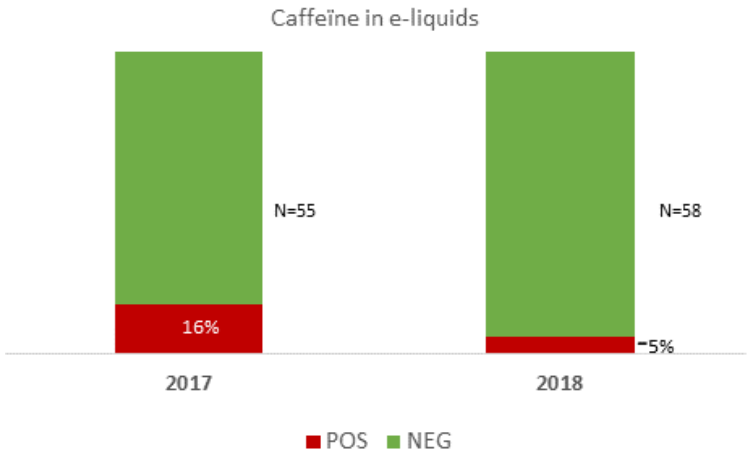

Figure 7.4: *Caffeine in e-liquids (no caffeine present (NEG), caffeine present (POS)).*

Table 7.7: Caffeine concentrations (mg/ml) in e-liquid samples identified with caffeine.

Code	flavour	caffeine concentration (mg/ml)
0041-17-001	coffee	<LOQ
0041-17-002	coffee	<LOQ
2001-17-0009	tobacco	<LOQ
1206-17-004	sweet	<LOQ
1206-17-008	tobacco	<LOQ
2001-17-0018	tobacco	0.008
2001-17-0019	tobacco	0.008
2001-17-0020	sweet	0.008
2001-17-0017	tobacco	0.009
1202-18-0224	coffee	0.021
1301-18-0195E	coffee	0.029
1202-18-0225	coffee	0.029

3.5 Harmful ingredients: Diacetyl and Acetylpropionyl

The amount of diacetyl and acetylpropionyl, putatively present in 40 sweet-flavoured e-liquids (2016-2018), was assessed by HS-GC-MS. As shown in Figure 7.5, diacetyl was present in 11 samples, acetylpropionyl in two samples and both compounds were simultaneously present in two sweet flavoured samples [45]. It was noticed that the positive e-liquids belong mainly to the brown flavours (e.g. caramel, chocolate and coffee-associated flavourings). The positive samples were further

quantified and the concentrations varied from 5-287 µg/ml and 31-115 µg/ml for diacetyl and acetylpropionyl, respectively (Table 7.8).

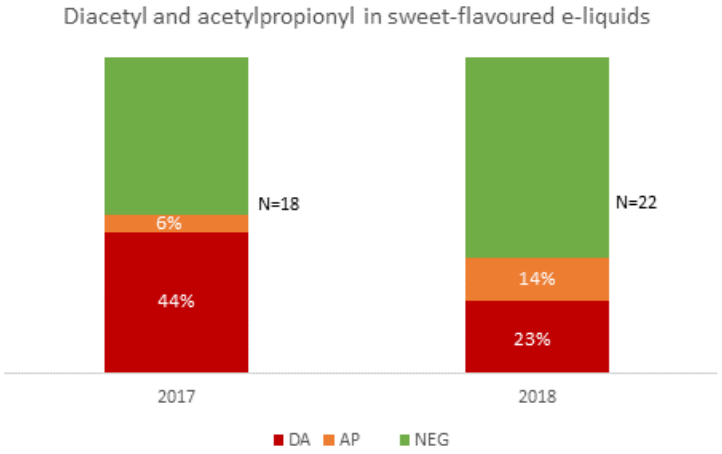

Figure 7.5: *Diacetyl (DA) and acetylpropionyl (AP) in sweet-brown flavoured e-liquids.*

Table 7.8: Concentrations (µg/ml) of diacetyl and acetylpropionyl in the e-liquid samples that were positively screened for both components

Code	Category	Diacetyl (µg/ml)	Acetylpropionyl (µg/ml)
1202-18-0233	Chocolate	-	31
1206-17-004	Nuts	-	71
1206-17-011	Nuts	82	-
2001-17-0018	Tobacco/caramel	5.1	-
2001-17-0019	Tobacco/caramel	7.7	-
1301-18-0198E	Cake	7.6	-
1202-18-0243	Sweet/cake	5.4	-
327242-S3	Cake	98	-
2001-17-0008	Tobacco/caramel	65	-
2001-17-0009	Tobacco/caramel	6	-
2001-17-0006	Sweet/cake	18	-
2001-17-0015	Sweet/cake	33	-
0040-17-004	Tobacco/nutty	287	-
1202-18-0234	Tobacco/caramel	33	43
1202-18-0220	Chocolate	<LOQ	116

3.6 Nicotine - stability study

The concentration of nicotine was analysed during 9 months in the different spiked e-liquids. At a first glance, nicotine seems stable in all matrices (Figure 7.6). However, an increase in the impurities nicotine-N-oxide and myosmine, was observed over time (Figure 7.7). A possible explanation for this rather contradictory results is the fact that the used UHPLC-DAD method is sensitive enough to quantify nicotine-impurities with high sensitivity and precision in the lower concentration ranges

(µg/ml), while the precision and thus the power of the method to detect significant small differences in the higher concentration range (mg/ml) of nicotine concentration is rather low. The nicotine-N-oxide concentration exceeds the Ph. Eur limit after 3 months, while myosmine remains within the limits of specified impurities. This effect is more pronounced in a 1,3-propanediol e-liquid matrix. No effect was observed on nicotine stability when the e-liquid samples were exposed to light.

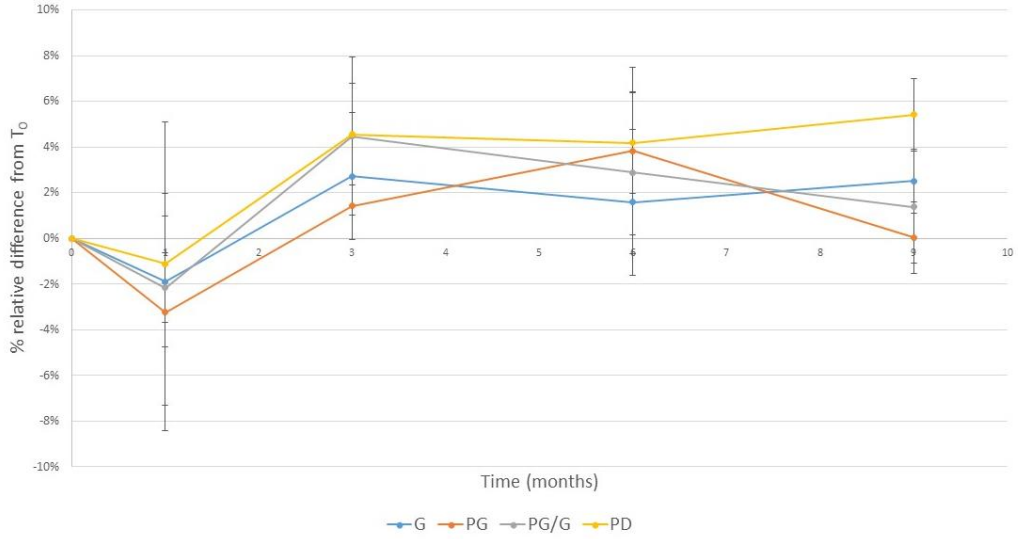

Figure 7.6: *Stability of nicotine in e-liquid matrices. The relative difference of nicotine at 1,3,6 and 9 months versus the initial concentration ± SD is shown for the different matrices. As there was no significant difference between samples stored in the dark or exposed to light, the pooled averages ± SD are shown. G = glycerol, PG = propylene glycol, PD = 1,3-propanediol.*

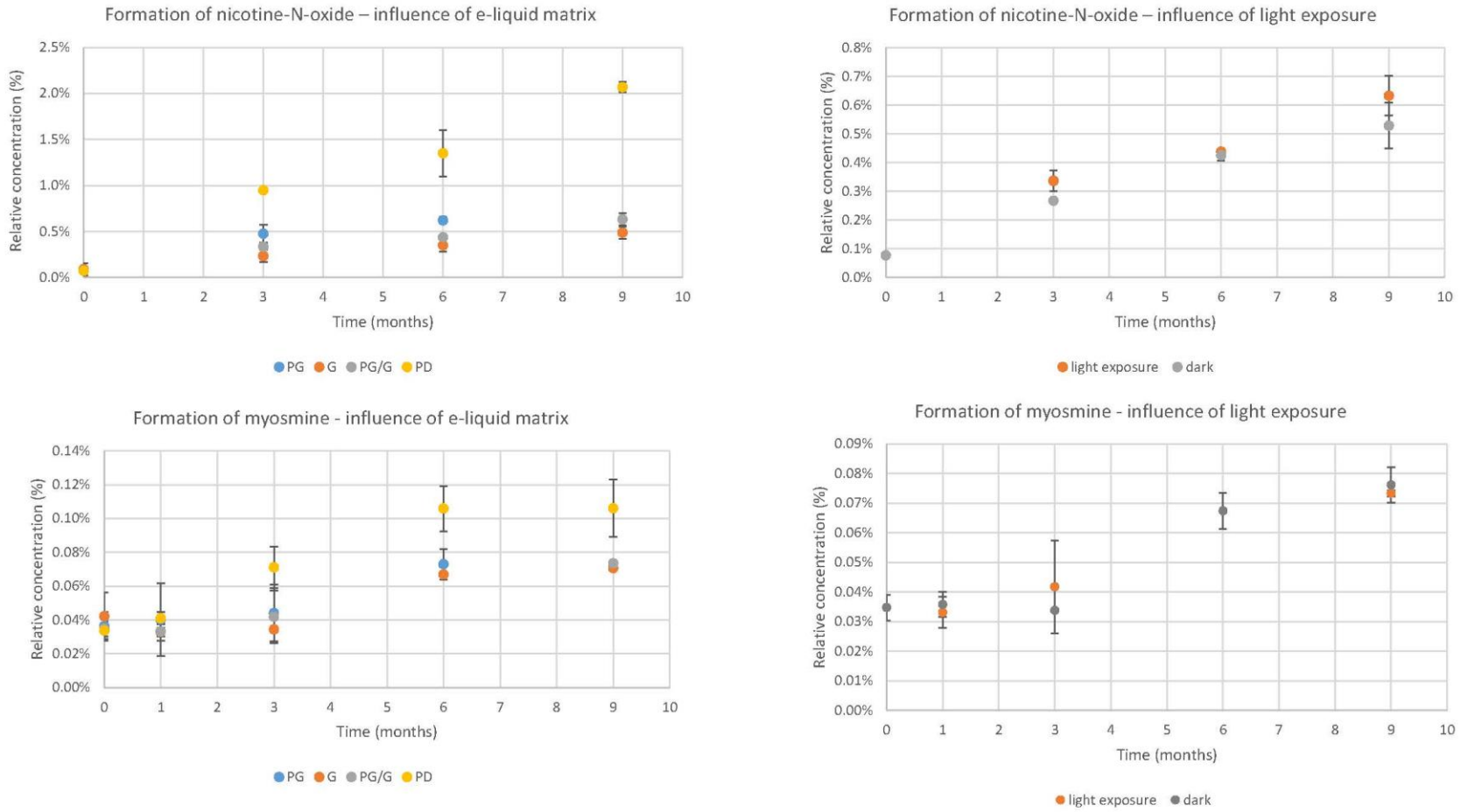

Figure 7.7: *Formation of nicotine-related impurities in e-liquid matrices. Left: The influence of the different e-liquid matrices on the formation of nicotine-N-oxide and myosmine for propylene glycol (PG), glycerol (G), PG/G and 1,3-propanediol (PD). Right: The influence of light exposure on the formation of nicotine-N-oxide and myosmine for the PG/G matrix.*

4 DISCUSSION

Similar to pharmaceutical ingredient content analysis in medicines, **nicotine** content analysis is seen as a quality indicator for e-cigarettes. Our data suggest that labelling discrepancies have decreased over time. Nevertheless, not all manufacturers manage to manufacture and label their products correctly. Label discrepancies might have several causes. The "zero-liquids" found with nicotine traces are probably due to contamination during manufacturing and handling. The presence of quantifiable amounts of nicotine (>1mg/g) is, however, more likely because of mislabelling. Several studies also suggest that poor storage conditions or unstable e-liquid formulations can lead to degradation of nicotine [33]. This could explain the observation of e-liquids with a nicotine concentration lower than the claimed concentration. However, in our 9 month stability study the nicotine concentration did not vary more than 10% of the initial concentration making this explanation unlikely.

From a regulatory point of view, zero-liquids that contain traces of nicotine do not comply with the TPD labelling criteria. However, the biological effects and potential associated harms due to exposure to these nicotine traces have not yet been assessed. The effect of the nicotine e-liquid concentration on the nicotine exposure by vaping is not as significant as other vaping parameters (device, vaping behavior,…) [31]. Hence, poor labelling accuracy of nicotine-containing e-liquids is more a matter of misleading the consumer about the actual nicotine content than it is a safety concern as the impact of inhaling a lower or slightly higher nicotine dose is unlikely to be harmful for a nicotine addict [31]. However, it has been suggested that even these small nicotine label discrepancies might be of significant concern when teenagers (<18 years) and persons trying to quit their nicotine addiction are unintentionally exposed [75].

According to the TPD, avoidable impurities should be limited in e-liquid products [3]. Therefore, next to the quantity of nicotine also its quality was investigated. Nicotine can either be extracted from the tobacco plant or chemically synthesized. The latter is the most expensive and to our knowledge not often used for e-liquids. Yet, due to the nicotine extraction from tobacco plants, **tobacco-related impurities** are unavoidable in the nicotine extract.

The presence of nicotine-related impurities in e-liquids is also linked to the stability of nicotine in the e-liquid matrix. Previous stability studies showed results comparable to ours for the impurity nicotine-N-oxide. Liu et al (2017) showed that the formation of nicotine-N-oxide is temperature and humidity dependent [76]. Flora et al. (2016) conducted stability studies up to 6 months on nicotine cartridges packed in blisters. A significant increasing trend could be observed for nicotine-N-oxide, nornicotine, myosmine and cotinine, though the found concentrations were not as pronounced as observed in our stability study [46]. The different results between the studies can probably be explained by the final

packaging in which the e-liquid is stored. In the study of Flora et al. cartridges are stored in a blister packaging with back foil which protects against external factors such as humidity and light and is also more airtight compared to the in-house packed samples that were used in our stability study.

The presence of nicotine-N-oxide is unavoidable in an e-liquid matrix because of the N-oxidation of nicotine that occurs spontaneously over time. The biological effects of nicotine-N-oxide are not extensively studied. It is, however, known that it is a major metabolite of nicotine that is reduced back to nicotine in the body, and thus leads to recycling of nicotine [77]. Myosmine is not only found in tobacco plants, but is also found in nuts, cereals and other foods [78]. The myosmine concentrations found in the investigated e-liquids were, however, in the relatively higher ranges (µg/g) as compared to concentrations found in food (ng/g). Unlike the other tobacco-related impurities, myosmine is reported as a potential genotoxic compound [79]. Therefore, whilst investigating the potential genotoxicity of this component in more detail, it should be avoided in e-liquids.

One should note that the majority of the e-liquid samples did not contain the impurities anabasine, anatabine, cotinine and nornicotine and that these impurities were not formed over the time of 9 months in our stability study. Nevertheless, these impurities are also an interesting indicator of the quality of the e-liquid. Thus, manufacturers should take measures to constrain the presence and the rapid formation of these nicotine-impurities in e-liquids and as well provide well-supported expiration dates on their products so that the consumer is sure that the quality of their product complies to the TPD.

Other impurities that can be avoided are the **VOCs**. Ethanol is a VOC that is intentionally added in e-liquids and listed as ingredient. Next to propylene glycol and glycerol ethanol is considered as one of the constituents of an e-liquid base. It was identified in the majority of the investigated e-liquid samples (data not given). Ethanol will not be further discussed as a volatile impurity though it should be noted that the effects of inhaling ethanol are not that innocent and more research is needed to assess the risk of long-term ethanol inhalation through e-cigarettes [80]–[82]. Another VOC intentionally added to e-liquids is ethyl acetate, which is also listed as a residual solvent Class 3 in the ICH guidelines [83]. Ethyl acetate is known as a flavouring component and can be found in various alcoholic beverages. In the context of e-cigarettes, it is necessary to assess the risks associated with its exposure through inhaling. However, as it is intentionally added as a flavouring agent and ethyl acetate is currently not known as inhalation toxicant by the competent authorities, e-liquids containing ethyl acetate are compliant with the TPD. We specifically investigated VOCs that are not intentionally added. High risk VOCs such as naphthalene, ethylbenzene and xylene were present in the investigated samples in concentrations well below the maximum allowed limits for pharmaceutical

products [83]. Isopropanol, however, was present in concentrations above the ICH limit, but below the oral toxicity limits [84], nevertheless they also exceed the current airborne occupational hazard limit (400 ppm) [85]. Other VOCs with a higher toxicity profile, including benzene and toluene, were not found in the investigated samples, contrary to previous reports [64], [86]. These previous studies indicated an overall higher percentage of positive samples for VOC, with a concentration range similar to our findings. The VOCs detected in e-liquids are mainly contaminants of the used ingredients such as the nicotine and flavouring extracts, which might implicate doubtful manufacturing practices. This should be limited by using high purity ingredients as required by the TPD. Next to the presence of these VOCs in the e-liquids, some suggest that VOCs could be formed by the heating process during vaping of the e-cigarette [87]. Therefore VOCs are contaminants that could be found in higher concentrations in the e-cigarette vapours compared to the e-liquid.

Diacetyl and **acetylpropionyl** are two controversial flavourings used in e-cigarettes. Both are examples of flavours that are generally considered as safe in food, but their inhalation is reported to be associated with respiratory inflammatory diseases. The investigated samples were purchased after 2016 i.e. when the attention was drawn to the potential inhalation toxicity of both components. From our analysis, it can be seen that there is a positive evolution ongoing, as the number of positive samples for diacetyl and acetylpropionyl was lower compared to samples from previous studies [13], [68] where respectively 90% and 75% of the samples contained one of both diketons. Compared to our study, we found that in 2017, 9 samples out of the 18 sweet flavoured e-liquids (50%) were either positive for diacetyl, acetylpropionyl or both, while in 2018 this was the case for 6 out of 22 sweet flavoured samples (27%). More recently, LeBouf et al. found that 70% of the brown flavours contained either diacetyl or acetylpropionyl [64]. Evidence for inhalation toxicity of diacetyl and acetylpropionyl remains a point of discussion [88]. Diacetyl is suggested to cause bronchiolitis obliterans, a rare disease typically found in popcorn workers after long-term and repeated exposure. It has been difficult to proof that diacetyl was the culprit, as the mechanism of toxicity is still not completely unraveled and the exposure threshold above which physiological changes of the airways are observed is high [89]. The lack of short-term toxicity and the high LD50 value also give the perception that health risks related to the presence of diacetyl and acetylpropionyl in e-cigarettes are limited. The TPD does not explicitly prohibit the use of these flavourings. In fact, the TPD is unclear about flavours used in e-cigarettes. To assure the safety of consumers, a next step in the regulation of e-cigarettes should therefore encompass inhalatory risk assessment of flavourings in order to compile lists of restricted/prohibited flavouring substances.

Similar measures were taken to regulate the use of additives in e-cigarettes such as **caffeine** and **taurine**. The main reason of the prohibition of caffeine and other stimulants in e-liquids in the EU is that they might give the perception that these e-cigarette products may be used for lifestyle purposes instead of nicotine replacement therapy. Consequently, they could also contribute to the normalization of cigarette use, although this has not been investigated yet. In our study, caffeine was detected in quantifiable concentrations in 7 out of 112 samples (6% of the samples). A previous study investigated the presence of caffeine specifically in coffee, tea, chocolate, and energy drink flavoured e-liquids and e-liquids that claimed to contain caffeine [90]. Indeed caffeine has been found in various e-liquid samples and in much higher concentration: 13 out of the 31 'caffeinated' flavoured samples contained caffeine. We included 25 caffeine-associated flavoured e-liquids of which only five contained caffeine. The presence of caffeine in those e-liquids can be explained by the use of natural coffee extracts as e-liquid flavourings instead of artificial coffee flavourings. In this study, however, not only caffeine-associated but also other different flavours have been included. Interestingly, in 5 out of 14 samples of a particular brand with non caffeine-associated flavours, caffeine was detected in concentrations of 2 µg/ml to 8 µg/ml. All these samples were tobacco-flavoured e-liquids; the presence of caffeine in these e-liquids remains unclear. These e-liquids are manufactured in Europe for the European market, thus contamination from e-liquids intended for the non-EU market is rather unlikely.

The potential health risks of the inhalation of caffeine through e-cigarettes are currently unknown. These do not only depend on the found concentration in the e-liquids, but also on the amount that is actually transferred from the e-liquid to the vapour and in particular on the local effects of caffeine on the respiratory tract and lungs.

Taken together, our study results clearly demonstrate that the implementation of the revised TPD has improved the quality of the e-liquids on the Belgian market. There are, however, certain limitations that could be addressed in future research. First, due to insufficient amounts, not all e-liquid samples could be investigated for each component. Also, a follow-up of e-liquids of the same brand before and after the implementation of the TPD would allow an even more accurate conclusion, however this was not possible because the brands analyzed before the TPD were not available on the Belgian market after the implementation of the TPD. The third limitation concerns the restriction of the quality parameters chosen, namely the levels of nicotine, nicotine-related impurities, VOCs, additives caffeine and taurine, and the flavours diacetyl and acetylpropionyl. Although these parameters are the most important ones, including also nitrosamine and heavy metals impurities would have been even more complete, but requires yet other analytical instruments.

Lastly, this study only focused on the e-liquids. Emission studies on e-cigarette aerosols would, however, have made it also possible to perform a preliminary safety evaluation of the poor-quality e-liquids identified.

5 CONCLUSION

E-cigarettes are not a new phenomenon anymore, nevertheless hitherto there was no overview of the quality of the different e-liquids available on the market. In this study, the major aim was to assess the compliance of currently available e-liquids to the TPD by analysis of the nicotine, nicotine-related impurities, VOCs, additives caffeine and taurine and the flavours diacetyl and acetylpropionyl levels. In addition, we also investigated whether the recent changes in the TPD have affected the quality of e-cigarette liquids by testing samples acquired from specialized vapeshops as well from the internet before, during and after the implementation of the revised TPD.

Our results demonstrate that since the implementation of the revised TPD the quality of zero-liquids has improved. However, nicotine label discrepancies are still common. Therefore continuous monitoring of the e-liquid market remains important, together with measurements to assure good e-liquid products (in the context of harm reduction) such as the use of high quality starting materials, good manufacturing practices and stability testing for appropriate storage packaging. Thus, overall the quality of the e-liquids nowadays are better than before the implementation of the TPD. Vapeshop samples are generally more compliant to the TPD than internet samples and DIY samples. These findings support the Belgian legislation to retain the prohibition of the internet sale of e-liquids.

It has to be mentioned that these results only reflect the e-liquids itself since the aerosols of e-cigarettes were not investigated. Indeed, there are many aspects that still need to be explored: the process of heating, the interactions between the different components, the formation of other (hazardous) components, inhalatory toxicity studies and the effect of mixture toxicology. Thus, while we can state that the analytical methodologies for chemical characterization are widely established, the standardization of emission studies is still in its infancy. Therefore, considerably more work needs to be done for the harmonization of emission studies of e-cigarette aerosols.

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PART III: A TOXICOLOGICAL ASSESSMENT OF E-CIGARETTES LIQUIDS

CHAPTER VIII - RISK ASSESSMENT OF E-CIGARETTES: A CASE STUDY OF DIACETYL

From the market study described in Chapter VII, it is clear that the flavouring components diacetyl and acetylpropionyl are present in e-liquids, despite concerns for inhalation toxicity. Yet, according to the current regulatory framework these compounds are not banned in e-liquids. Hence, there is a need to more thoroughly investigate whether these flavours (and other compounds) actually pose a health risk in the concentrations present in e-cigarette refills and to which the user is exposed. Therefore in this chapter, a methodology to assess the local and systemic health risks for inhalation toxicity of e-liquid components is proposed based on the case of diacetyl.

1 INTRODUCTION

In general, the risk assessment process, in relation to human health is a three-step process including the hazard assessment (= hazard identification and characterization), the exposure assessment and the final risk characterization.

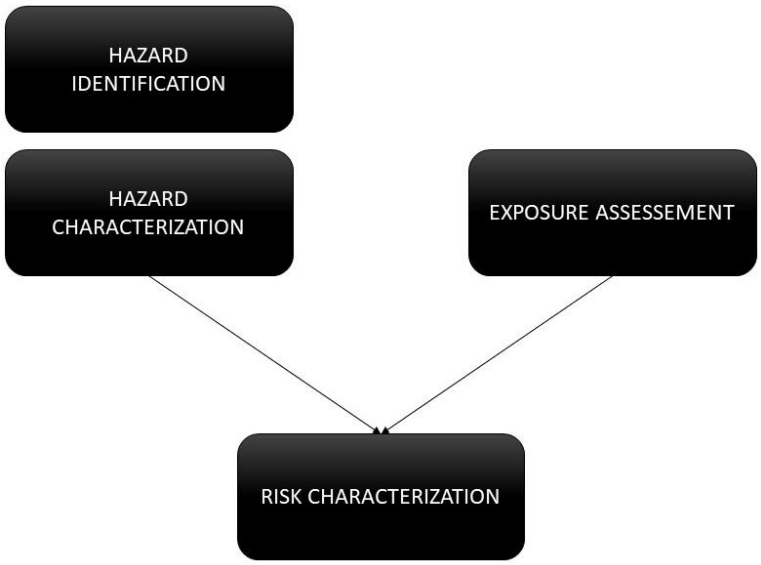

Figure 8.1: *Schematic overview from the principle of risk assessment.*

The hazard identification is to determine whether a substance has the ability to provoke specific adverse health effects. It is the determination of the intrinsic properties of that substance independent of dose or exposure. In the context of e-cigarettes, it will be necessary but also a challenge to identify the potential adverse health effects for each individual constituent present in the e-liquid. The hazard characterization or dose-response assessment is to determine the level below which the identified toxic effects are not expected to occur. It answers the question of what dose is necessary to cause harm. Next, the exposure assessment includes an estimation of the

concentrations/doses to which humans are or may be exposed in this case via e-cigarettes. When these three pillars are combined, characterization of the risk can be performed (Figure 8.1) i.e. estimation of the incidence and severity of the adverse effects likely to occur in humans due to actual or predicted exposure to a substance. Basically, the process of risk assessment of a component consists of comparing the concentration of that component to which humans are (likely to be) exposed with the concentration at which no toxic effects are expected to occur.

Risk assessment for human health addresses the different potential toxic effects in different human populations, considering the different exposure routes (Table 8.1). For e-cigarettes, we will specifically focus on the general population risk for acute and repeated dose toxicity through inhalation. Both local and systemic toxicity will be considered.

Table 8.1: Different variables in risk assessment; population, toxicological endpoint, exposure route and frequency of exposure

Population	Toxic effects	Exposure route	Frequency of exposure
general population workers	acute toxicity irritation corrosivity sensitisation repeated dose toxicity mutagenicity carcinogenicity toxicity for reproduction	oral dermal inhalation	acute subacute sub-chronic chronic

The exposure through inhalation can occur intentionally e.g. as the result of vaporizing solutions or aerosol-forming mixtures (sprays, perfumes, etc.) or non-intentionally as a result of volatilization e.g. evaporation of solvents from paints or occupational hazards in manufacture plants. Exposure by inhalation is usually expressed as the average concentration of the substance in the inhaled air over a reference period of time, which will normally be the duration of exposure [1]. In that regard, the exposure time for the use of an e-cigarette is rather irregular.

Because exposure via e-cigarettes is a dynamic process encompassing many variables, it is challenging to perform a risk assessment for substances found in e-liquids and in the aerosol of e-cigarette emissions. The first challenge is the difference in the use of e-cigarettes between individuals, which can vary according to the different purposes [2]. People trying to quit smoking will use their e-cigarettes as little as possible, while the so called “cloud chasers” or people for which the e-cigarette has become a part of their lifestyle/identity, will vape more frequently and use higher amounts during

the day. Therefore, most assessments apply a tiered approach where the risk is assessed for a worst case scenario i.e. the use by heavy vapers.

The exposure not only depends on the users' behaviour, but also on the type of e-cigarette device, the concentration of the respective substance in the e-liquid, the duration of exposure, the frequency of vaping sessions and the number of puffs within a vaping session. The number of official studies and surveys on e-cigarette use and topography is, however, scarce. The latest surveys date from 2014 by the RIVM and Farsalinos et al. and involve mainly second generation devices [3], [4].

In this chapter, a case study is presented which shows the risk assessment for diacetyl in the concentration measured in e-liquids. As shown before, both diacetyl and acetylpropionyl are controversial flavourings, that should be avoided because of their potential to cause bronchiolitis obliterans [5]. Consequently, the risk for (local) respiratory irritation and inflammation after repeated dosing is assessed for the highest concentration of diacetyl measured in e-liquids. The risk for systemic toxicity of diacetyl exposure through repeated e-cigarette vaping is also addressed. The methodology proposed by the RIVM and described by Bos et al. [6] for tobacco components and smoke additives has been used. The calculations used in this chapter are based on the model described by Visser [3]. This model is briefly discussed in the next paragraph.

2 INHALATION TOXICITY RISK ASSESSMENT

2.1 Local inhalation toxicity

Studies suggest that diacetyl provokes both local and systemic effects [7]. In this paragraph we will focus on the risk assessment for local toxicity effects. A local effect is observed at the site of first contact and it is caused irrespective of the fact whether a substance becomes systemically available or not. Local effects may be a minor effect such as irritation that is reversible and characterized by redness and oedema, but they may be irreversible major effects such as corrosion and massive cytotoxic effects. Dose-response assessment characterizes the hazard and shows the relationship between the dose and the toxic effect(s). A reference point or 'point of departure' (POD) is derived from this dose-response relationship, which is usually obtained through *in vivo* animal studies. For local effects, the POD_{local} is the so-called local No Observed Adverse Effect Concentration (NOAEC) or the highest exposure concentration at which a test chemical does not induce an adverse effect in the test animal. A local effect can occur either after a single dose or after a repeated dose exposure, and needs to be taken into account when defining the POD_{local}. For local inhalation toxicity, the NOAEC is preferably derived from investigations where the route of exposure is inhalation as well. However, most inhalation studies involve exposure of the animals to the compound under investigation in a

specific chamber to a well-defined concentration of the substance in the air for a well-defined time period. This scenario where inhalation occurs when the animal is in rest, is used for occupational risk assessment. Here, an alternative methodology is proposed to assess the risk as it concerns intentional inhalation during a vaping or smoking session which is not only shorter but also involves higher concentration pulses. Therefore, the POD_{local} used here, is derived from an internal NOAEC which has been established earlier from an external NOAEC of animal studies. How these values are obtained, will be demonstrated using diacetyl as a case study. Next, this internal NOAEC will be compared to the internal dose of the substance to which the e-cigarette user is exposed. Therefore, the exposure via e-cigarette inhalation will be assessed by calculating the maximum alveolar concentration of the substance obtained after a repeated number of puffs that are representative for an average vaping session (Figure 8.2).

2.1.1 Exposure assessment

The exposure assessment includes two steps as shown in Figure 8.2. First, the concentration in the aerosol is estimated in order to determine the maximum alveolar concentration to which the respiratory tract is exposed after inhalation (repeated or single dose).

2.1.1.1 Estimation of the aerosol concentration

The alveolar concentration is derived from the diacetyl concentration in the aerosol, which will be estimated from the amount of diacetyl measured in the e-liquids. Even more accurate would be to experimentally determine the concentration of diacetyl in the aerosol. However, the appropriate technique for emission studies was not at our disposal in the host laboratory. Nevertheless, diacetyl is highly volatile and is therefore completely transferred from the e-liquid into the aerosol phase. In addition, there is a strong correlation between the expected and measured diacetyl values in the emissions. Thus, diacetyl is readily delivered from the liquid to the aerosol and no additional diacetyl is produced during the evaporation process [8].

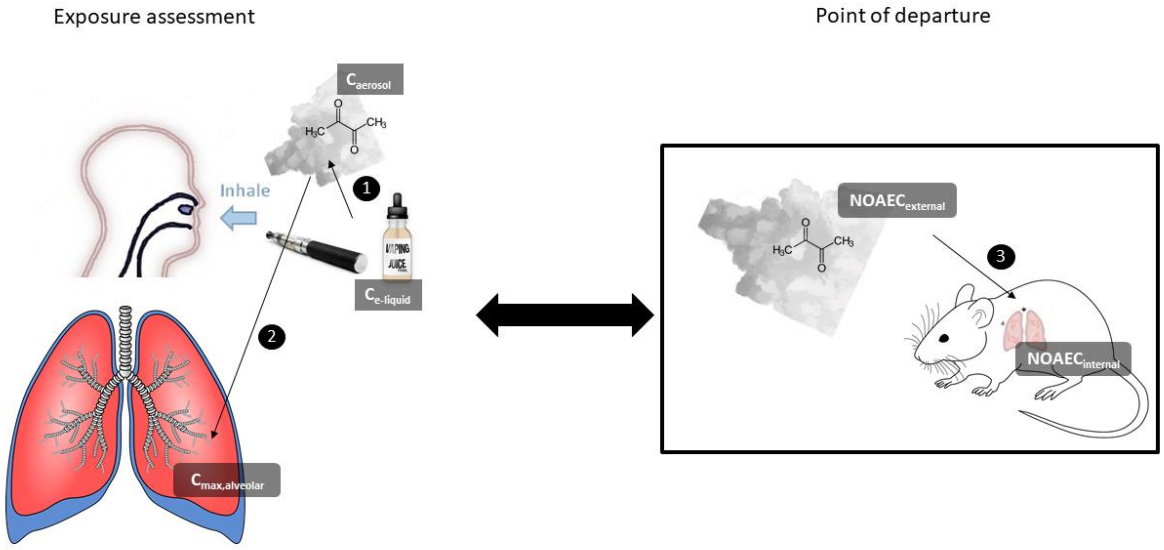

Figure 8.2: *Overview of risk assessment for local toxicity via inhalation. The exposure assessment (left) includes (1) the estimation of the aerosol concentration and (2) the estimation of the concentration in the respiratory tract ($C_{alv,max}$). For the POD_{local} (right) the NOAEC needs to be converted to an internal NOAEC (3).*

Hence, the amount (mg) of e-liquid that is transferred to a certain volume of aerosol (ml) has to be determined in order to estimate the diacetyl concentration in the e-cigarettes emissions.

$$C_{aerosol} = \frac{\frac{C_{e-liquid}}{\rho_{e-liquid}} \times m_{e-liquid,puff}}{V_{puff}} \times 1000 \qquad (1)$$

$C_{aerosol}$	= concentration of the substance in the aerosol (µg/ml)
$C_{eliquid}$	= concentration of the substance measured in the e-liquid (g/ml)
$\rho_{eliquid}$	= density of the e-liquid (g/ml)
$m_{eliquid,puff}$	= e-liquid consumption/puff (mg/puff)
V_{puff}	= puff volume (ml)

The first three parameters ($C_{aerosol}$, $C_{eliquid}$, $\rho_{eliquid}$) are depending on the investigated e-liquid. For the last two parameters (e-liquid consumption/puff and puff volume) no default values are available in the literature as these are highly variable depending on different parameters such as the duration per puff, the voltage of the battery, the type of device (2nd vs 3rd generation) and the PG/G/flavour composition of the e-liquid. For the 2nd generation devices (eVOD) our estimations are based on RIVM

data [3] where the amount of consumed e-liquid per puff for a battery voltage of 3.7V and 4.8V has been set at 4 mg/puff and 8 mg/puff, respectively, and one puff has a set volume of 70 ml (Table 8.2).

Table 8.2: Puff volume and e-liquid consumption. Data from [3].

Puff volume (ml)	70
Lowest e-liquid consumption/puff at 3.7V (mg/puff)	4
Highest e-liquids consumption/puff at 4.8V (mg/puff)	8

2.1.1.2 Estimation of the concentration in the respiratory tract ($C_{alv,max}$):

To estimate the alveolar concentration, further dilution of the aerosol concentration in the respiratory tract needs to be taken into account (step 1). Moreover, because vaping is an intentional inhalation of consecutive puffs, the initial alveolar concentration will increase until a stabilized maximum alveolar concentration is reached. This concentration to which the respiratory tract is exposed is based on the specific topography of vaping (step 2).

Step 1. Estimation of the initial alveolar concentration based on the inhaled aerosol concentration

The initial alveolar concentration is calculated from the concentration of the component in the inhaled aerosol. The first dilution is the dilution of the aerosol concentration of a puff when it is mixed with the the air volume that is inhaled during breathing at rest, i.e. the so-called tidal volume (Figure 8.3).

$$DF_1 = \frac{TV + V_{puff}}{V_{puff}} \quad (2)$$

DF_1 = dilution factor 1 i.e. dilution of puff volume with tidal volume

TV = tidal volume (ml)

V_{puff} = puff volume (ml)

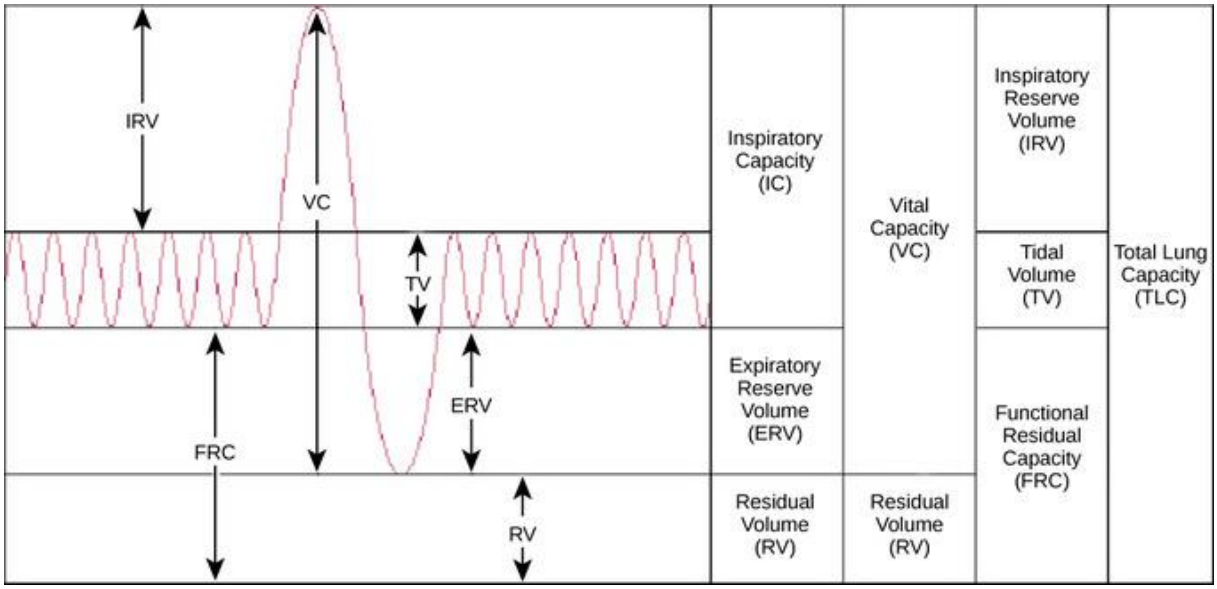

Figure 8.3: *Definitions of lung volumes and capacities [8].*

A 4-sec puff of 70 ml is taken and the e-cigarette aerosol is inhaled, together with a volume of clean air (= the tidal volume at rest of 500 ml). Consequently, the concentration of components in the e-cigarette aerosol will decrease by a factor of 8 (570 ml/70 ml) (Table 8.3).

The second dilution occurs when the concentration in this inhaled air is further mixed with the clean air of the whole human lung capacity i.e. functional residual capacity (FRC) (Figure 8.3). During a breathing cycle, approximately 30% of the inhaled volume of air (= the dead space volume) does not reach the alveoli where the final gas exchange with the blood takes place. The calculations are as follows:

$$DF_2 = \frac{V_{inh}}{FRC + V_{inh}} \quad (3)$$

$$V_{inh} = \left(TV + V_{puff}\right) \times (100\% - DSV\%) \quad (4)$$

DF_2	= dilution factor 2 i.e. dilution of inhalation volume with total volume in respiratory tract (FRC)
FRC	= functional residual capacity
V_{inh}	= inhaled volume (mL)
TV	= tidal volume (ml)
DSV%	= dead space volume %

Thus only 70% of the inhaled 570 mL (e.g. 400 mL) reaches the alveoli. This volume of 400 mL is mixed by diffusion with the volume of air present in the lungs (i.e. functional residual capacity (FRC) which equals approximately 2 L). Therefore, the concentration of e-cigarette aerosol substances is diluted further by a factor of maximally 6 (2400 mL/400 mL). This is without taking into account the actual process of diffusion, which is slower and presumably not completed before the next cycle of inhalation. Because of that, the distribution within the lungs is uneven with varying concentration. Therefore, for the estimation of the initial alveolar concentration of a component an arbitrary factor of 3 is chosen, instead of a dilution factor of 6. Taken together, it is estimated that the initial concentration of a component in alveolar air (Calv;initial) will be diluted with a total factor of 24 (8×3) (Table 8.3).

$$C_{alv,init} = \frac{C_{aerosol}}{DF_1 \times DF_2} \quad (5)$$

Table 8.2: Lung parameters necessary to calculate the initial alveolar concentration.

Volume single puff	70 ml
Tidal volume at rest	500 ml
Dead space volume	30%
Dilution 1	570/70 = 8
Final volume reaching alveoli	570 ml x (100% – 30%) = 400 ml
Functional residual capacity	2000 ml
Dilution 2	2400/400 = 6 → corrected for incomplete diffusion to a default value of 3
Total dilution	24

Step 2. Estimation of the maximum alveolar concentration based on the topography of vaping

The estimation of the maximum alveolar concentration is based on the complex and dynamic process involved in using an e-cigarette. As such, after one puff, the concentration of the e-liquid component in the respiratory tract will be relatively high, followed by a decrease in concentration due to normal breathing of clean air, thereby causing a dilution effect. After the next puff, the concentration increases again. The decrease of the concentration in the respiratory tract can be predicted as follows: the volume of air that is inhaled during breathing at rest (tidal volume) is 500 ml (Figure 8.3). Of this volume 70% reaches the deep respiratory tract (350 ml) where the volume of air is mixed with the functional residual capacity of the volume of air that remains in the lungs after exhalation during normal breathing (2000 ml). Thus, with each breathing, the concentration of the component will be decreased by 15% (350/2350).

This allows to calculate the maximal alveolar concentration during a vaping session. The decrease of the initial concentration not only depends on the time between each puff, but also on the absorption taking place at the alveolar level. The less absorption takes places in the alveoli, the higher the local concentration of the component in the respiratory tract will be. From a conservative point of view, the local toxicity on the respiratory tract will be assessed with an estimation of low absorption i.e. 30%. The maximal alveolar concentration is predicted from a simulation of a 6-minute vaping session where it is assumed that there is a 4-seconds puff, every 30 seconds, and a normal breathing frequency of 12 min-1 (Figure 8.4). After 5 puffs, a stable pattern is obtained where the initial concentration varies between a minimal concentration of 0.08 of the initial concentration and a maximum concentration of 1.1 relative to the initial concentration [3].

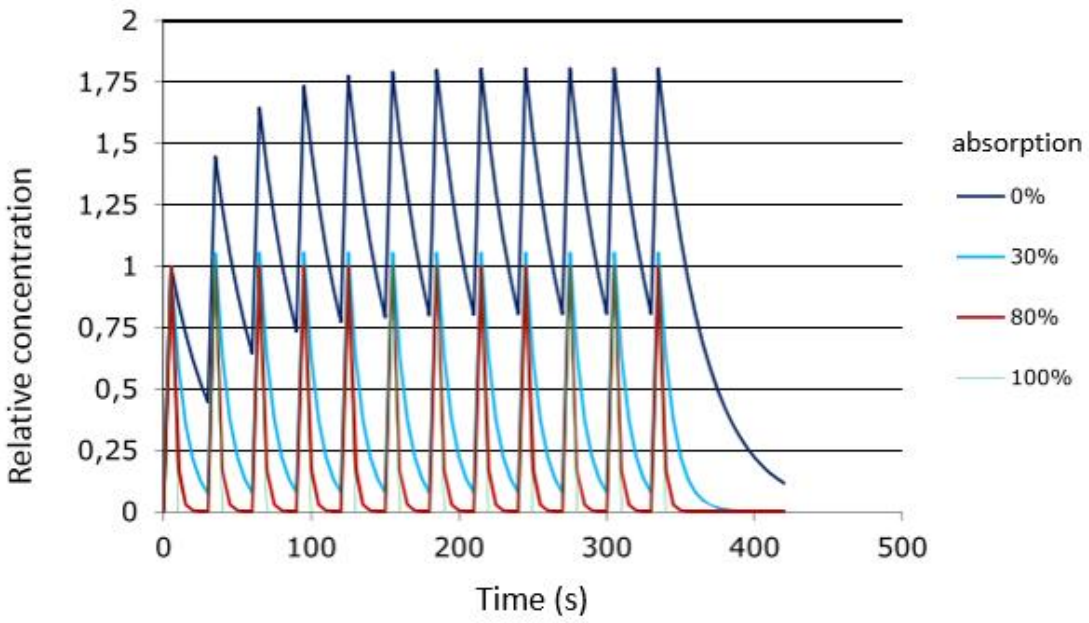

Figure 8.4: *Simulation of a 6-minute vaping session with a puff of 4 seconds each 30 seconds. Estimation of the relative alveolar concentration (initial alveolar concentration is 1). Adapted from [3].*

Thus, the maximal alveolar concentration of the component is calculated from its initial alveolar concentration. The initial alveolar component concentration ($C_{alv;initial}$) can be calculated using the equation (5). Under the assumption of 30% alveolar absorption, the maximal alveolar concentration during the use of an e-cigarette equals:

$$C_{alv;max} = 1{,}1 \times C_{alv;initial} = 0{,}046 \times C_{aerosol} \quad (6)$$

2.1.2 Point of Departure for local toxicity (POD_{local})

The dose-response assessment is established through a POD. This POD is in most cases obtained from studies in experimental animals conform to internationally agreed testing guidelines. Depending on which toxicity is characterized (acute, repeated dose, local irritation), the POD is selected from the corresponding animal study. Before the POD_{local} can be used for the dose response assessment and to compare with the actual exposure in humans, the NOAEC needs to be additionally converted into an internal NOAEC (2.1.2.1). The NOAEC is determined under certain experimental conditions, which are not representative for real-life conditions. Therefore, additional correction for these study parameters (exposure period, time of exposure) are applied (2.1.2.2).

2.1.2.1 Estimation of the internal NOAEC

Thus, for local toxicity of intentionally inhaled substances it is suggested to convert the external NOAEC obtained from animal studies to an internal NOAEC. As the external NOAEC is determined as a concentration of substance in ppm of the air in whole-body exposure chambers and different from the concentration to which the respiratory tract of the animals is exposed. Therefore, in order to compare with the $C_{max,alv}$ which is the concentration of the substance to which the respiratory tract is directly exposed, the $NOAEC_{external}$ needs to be converted. First for the conversion of the $NOAEC_{external}$ from ppm to mg/l the ideal gas law is applied. The unit (ppm) expresses a concentration in parts per million measured as the volume (denoted in liters [l]) of a substance found in 1l of a medium such as air.

$$\frac{mg}{L} = ppm \times \frac{MW}{22.4} \times \frac{273}{(273+T)} \times \frac{P}{1013} \quad (7)$$

MW	Molecular weight of a substance
22.4 (l)	The volume of 1 mol at 1 atmospheric pressure at 0 °C
273 (K)	add 273 to the Celsius ° value (273+T) to obtain the temperature in Kelvin
T	The room temperature of 20°C
1013 (hPa)	One atmospheric pressure (1atm)
P	denotes the atmospheric pressure at the point of measurement (hPa)

Next, the internal POD_{local} for inhalation toxicity is determined. Therefore, similar to the determination of $C_{max,alv}$, the $NOAEC_{external}$ is corrected for the dilution of the tidal volume with the functional residual capacity of the animal used in the study of the POD_{local} (Table 8.4). The equations (3) and (4) are applied with the lung parameters of the animals used in the studies from which the POD is derived. Only, the V_{puff} is not accounted for in equation (4).

The TV and FRC for male *Spague-Dawley rats* is 1.5 ml and 6.7 ml, respectively [9]. Thus, the inhaled concentration of diacetyl is further diluted by approximately a factor of maximally 7.4 (7.75 ml/1.05 ml*)* taking into account that only 70% of the TV reaches the deep respiratory tract (70% of 1.5 ml = 1.05 ml). The component will, however, not be evenly distributed within the lungs and the concentration will vary. Therefore, instead of a dilution factor of 7.4, an arbitrary factor of 3 is utilized for the estimation of the initial alveolar concentration of a component (Table 8.5).

The TV and FRC for male *C57BL/6 mice* is 342µl and 405µl, respectively [10]. Thus, the inhaled concentration of diacetyl is further diluted by approximately a factor of maximally 2.7 (645 µl/240 µl*)* taking into account that only 70% of the TV reaches the deep respiratory tract (70% of 342 µl = 240 µl). In summary, it is estimated that the alveolar air concentration of the component in (C_{alv}) will be a factor of 2.7 lower than the concentration in the inhaled air (Table 8.5).

Table 8.3: Lung parameters for laboratory animals potentially used in POD_{local} studies. The data for the male Spague-Dawley rats is retrieved from [9] and data for the male C57BL/6 mice from [10].

male Spague-Dawley rats	
TV (ml)	1.5
FRC (ml)	6.7
male C57BL/6 mice	
TV (µl)	342
FRC (µl)	405

2.1.2.2 Correction of POD_{local} for study parameters

The POD_{local} is additionally corrected for the differences in the exposure scenario between the animal study and daily use of e-cigarettes i.e. exposure duration of the animal study is 6 hours while in the scenario of a heavy vaper, the e-cigarette is used for 4 hours. Therefore the $NOAEC_{internal}$ for single and repeated exposures is multiplied by the ratio of the animal exposure condition versus the human exposure condition. Additionally, an extra correction is included for days of exposure: the 5 days exposure in the animals versus exposure during the whole week for vapers. For the repeated dose

study we add another correction for the duration of the study. These are often representative for subchronic exposure, while a factor is added to account for chronic exposure [1]. Thus, for the repeated dose toxicity a factor was accounted for the difference in duration of the animal study from subchronic to chronic exposure.

Table 8.4: Overview of the conversion and correction factors used to adjust the POD_{local}.

	Single administration, short term exposure	Repeated exposure
Conversion factors for $NOAEC_{external}$ to $NOAEC_{internal}$	3	2.7
Correction factors of POD for study parameters		
Correction of exposure time (4h instead of 6h)	1.5	1.5
Correction of exposure period (5 days/week instead of 7 days/week)	NA	0.71
Less-than-lifetime exposure present in the used animal experiment – subchronic to chronic	1	2
Total of correction factors	1.5	0.5325

2.1.3 Margin of exposure (MOE) for local inhalation toxicity

Once the assessment of the exposure and the internal POD_{local} have been established, one can estimate the risk, which is the probability that the harm will occur. This risk assessment is calculated as follows:

$$MOE = \frac{\text{internal POD}_{\text{local}}}{exposure} \quad (8)$$

The MOE should be higher or equal than the default value of 100, established by the WHO, in order to assure the safety of a substance. This factor 100 accounts for a factor 10 for intra-species differences (variability between humans) and another factor 10 for inter-species extrapolation (animal to human). However, because local effects are independent of the basal metabolic rate and the uncertainty is more based on toxicodynamic differences rather than toxicokinetics, a default factor of 2.5 is used instead of 10 for inter-species variation (Table 8.6). Thus, the risk exists if the MOE is smaller than 25.

Table 8.5: Assessment factors for the calculation of the MOE for local toxicity.

Assessment factors	Single administration, short term exposure	Repeated exposure
Intra-species	10	10
Inter-species variability (only toxicodynamics, not toxicokinetics because of local effects)	2.5	2.5
MOE	25	25

2.2 Systemic inhalation toxicity

The same three steps as for local toxicity effects will be followed for the risk assessment of systemic toxicity (Figure 8.5). For the dose-response assessment, the level below which systemic toxic effects are not expected to occur is determined. This is usually the no observed adverse effect level (NOAEL), historically derived through *in vivo* toxicity testing, and indicates the point of departure for systemic toxicity (POD_{sys}) of the substance under consideration. Before the risk can be characterized, an estimate of the systemic exposure dose (SED) is determined, i.e. the amount of the substance to which humans are actually exposed taking into account the duration and frequency of exposure, but also inhalation dynamics that are characteristic for the human respiratory tract. Finally, when the POD_{sys}/SED is equal to, or greater than, 100 (= default value set by WHO) the substance is generally considered safe under the intended use conditions.

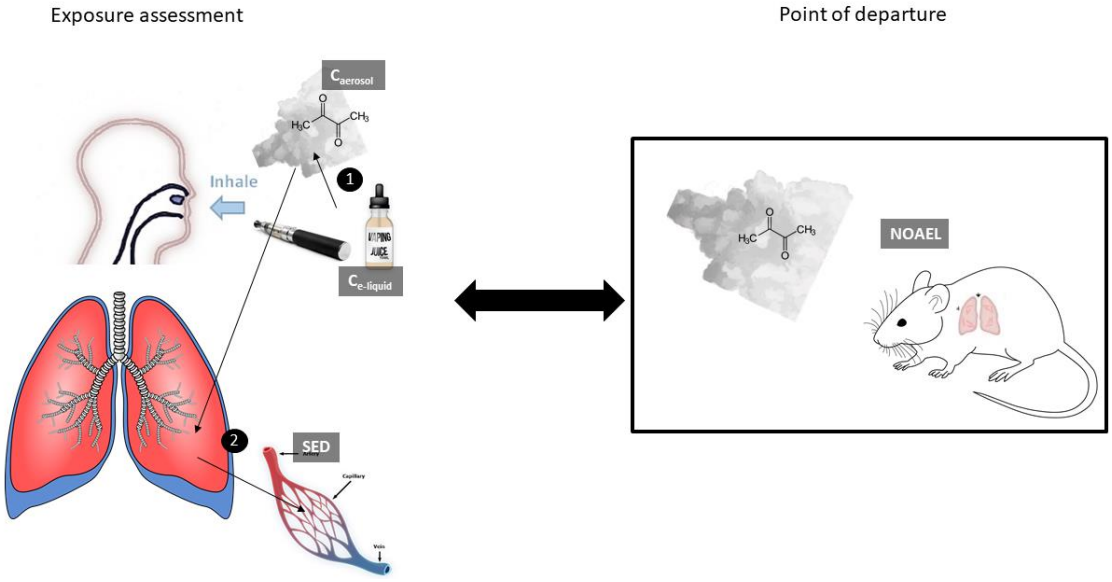

***Figure 8.5**: Overview of risk assessment for systemic toxicity via inhalation. The exposure assessment (left) includes (1) the estimation of the aerosol concentration and (2) the estimation of systemic exposure dose (SED). For the POD_{sys} (right) the NOAEL is obtained from in-vivo animal studies.*

2.2.1 Exposure assessment

For the exposure assessment the SED is determined. This is the total absorbed dose calculated from the daily e-liquid use and the concentration of the substance in the e-liquid. Thus, information about the daily e-cigarette use is required in order to calculate the total absorbed dose. In the survey of Farsalinos *et al.* in 2014, the amounts reported are between 2 and 5 ml daily [11]. However, nowadays the devices are more performant and liquid consumption is even higher. Yet, official information on the current use is not available. Therefore, the assessment is performed for the worst case i.e. daily e-liquid use of 5 ml. Next, we need to make two assumptions: (1) it is assumed that the test substance in the e-liquid will be 100% transferred into the aerosol emissions produced by the e-cigarette and (2) the absorption through the alveoli is 100%. Also, not all inhaled e-liquid reaches the deeper respiratory tract. During a breathing cycle, approximately 30% of the inhaled volume of air (the dead space volume) does not reach the alveoli where gas exchange with the blood takes place. Thus, maximally 70% of an inhaled dose can become systematically available. Thus, the SED can be calculated as followed:

$$SED = \frac{C_{e-liquid} \times V_{tot,daily}}{BW} \times (100\% - DSV\%) \quad (9)$$

SED = systemic exposure dose (mg/kg bw/day)

$C_{e\text{-}liquid}$ = e-liquid concentration (mg/ml)

$V_{tot,daily}$ = daily e-liquid consumption (ml)

BW = average body weight (kg)

DSV% = dead space volume %

2.2.2 Point of Departure (POD_{sys})

Analogously to the POD_{local}, the NOAEL is taken from a relevant study for comparison with the SED. Additional conversion to an internal NOAEL value is not necessary as we assume that the absorption through the alveoli is 100%. However, the NOAEL used for the POD_{sys} still needs to be corrected for the study parameters such as less-than-lifetime exposure of the animal experiment (subchronic to chronic) and the route-to-route extrapolation (oral to inhalation), if applicable.

Table 8.6: Overview of the conversion and correction factors of the POD_{sys}.

Correction of NOAEL for study parameters	
Less-than-lifetime exposure of the used animal experiment – subchronic to chronic	2
Route-to-route extrapolation – oral to inhalation	2

2.2.3 Margin of exposure (MOE) for the systemic effects of diacetyl

Analogous to the risk characterization for local toxicity, the MOE is calculated as the ratio of the POD_{sys} (NOAEL in this case) to the systemically absorbed dose per kg bodyweight per day. This value should be higher or equal than the default value of 100 set by WHO, which accounts for the inter-species extrapolation of rat to human (factor 10) and inter-individual variability (factor 10) [1].

Table 8.7: Assessment factors for the composition of the MOE for systemic toxicity.

Assessment factors	
Intra-species variability	10
Inter-species variability	10

3 RISK ASSESSMENT OF DIACETYL

The abovementioned methodology is applied to determine the inhalation toxicity of diacetyl in e-liquids as well for local as for systemic toxicity, more specifically to the highest amount of diacetyl found in e-liquids during our market survey which was 287 µg/ml e-liquid (see Chapter VII).

3.1 Local inhalation toxicity

3.1.1 Exposure assessment

3.1.1.1 Diacetyl concentration in the aerosol

Based on equation 1 and Table 8.2

Highest concentration diacetyl measured in e-liquid (mg/ml)	0.287*
Density of e-liquid (g/ml)	1.131
Highest concentration diacetyl in e-liquid (mg/g)	0.254
Lowest diacetyl aerosol conc (µg/ml)	0.014
Highest diacetyl aerosol conc (µg/ml)	0.029

* Experimental data obtained in Chapter VII.

3.1.1.2 Estimation of the concentration in the respiratory tract ($C_{alv,max}$):

Based on equation 2-6 and Table 8.3

Lowest diacetyl aerosol conc (µg/ml)	0.014
Highest diacetyl aerosol conc (µg/ml)	0.029
Max. alveolair concentration conversion factor	0.046
Lowest max. alveolair concentration (µg/ml)	0.0006
Highest max. alveolair concentration (µg/ml)	0.0013

3.1.2 Point of Departure

3.1.2.1 Local effects upon short-term exposure:

The NOAEC reported by Hubbs et al. is used as POD_{local} [12]. They investigated the acute inhalation toxicity of diacetyl vapour in rats at concentrations up to 365 ppm (time-weighted average (TWA)). One of the groups inhaled a single pulse of 1800 ppm diacetyl (which corresponds to 92.9 ppm in 6-

hour TWA) that caused epithelial injury, with the nasal epithelium being the most responsive. Therefore, it was concluded that the NOAEC for inhaled diacetyl was less than 92.9 ppm [12].

Critical endpoint	Histopathological changes in nasal epithelium and respiratory tract
Study	Hubbs et al, 2008 [12]
Species	Sprague-Dawley rats
Exposure regimen	Single pulse
Concentration tested (ppm)	< 365
Duration of exposure	Single administration
NOAEC (ppm)	92.9

Based on equation 7 and Table 8.4 and Table 8.5

External POD_{local} = $NOAEC_{external}$ (ppm)	92.9
Diacetyl conversion factor ppm → mg/l	0.00358
Converted $NOAEC_{external}$ (mg/l)	0.333
$NOAEC_{internal}$ (µg/l)	111
Corrected $NOAEC_{internal}$ (µg/l) = internal POD_{local}	167

3.1.2.2 Local effects upon repeated exposure:

For the POD_{local} at repeated dose the NOAEC reported by Morgan et al. is used [13]. In this study male C57BL/6 mice were exposed in a whole-body exposure chamber at diacetyl vapour levels of 0, 25, 50 or 100 ppm, 6 hours/day, 5 days/week for 6 or 12 weeks. Inflammatory changes, olfactory epithelial atrophy and metaplasia were observed in the mice exposed to 50 ppm diacetyl. Peribronchial lymphocytic inflammation was also seen in 4 of the 5 mice exposed to 50 ppm after 12 weeks of exposure. In 2 of the the 5 mice exposed to 25 ppm diacetyl, similar inflammation was observed, but in these animals it was minimal to mild and not accompanied by epithelial atrophy. The NOAEC for peribronchiolar lymphocytic inflammation was therefore set at 25 ppm. It was concluded that exposure to diacetyl vapour results in a pattern of injury that replicates features of human bronchiolitis obliterans [13].

Critical endpoint	Peribronchiolar lymphocytic inflammation
Study	Morgan et al, 2008 [13].
Species	Male C57BL/6 mice
Exposure regimen	6 hours/day, 5 days/week
Concentrations tested (ppm)	0, 25, 50, 100
Duration of exposure	6 or 12 weeks
NOAEC (ppm)	25

Based on equation 7 and Table 8.4 and Table 8.5

External POD_{local} = $NOAEC_{external}$ (ppm)	25
Conversion factor 1 ppm (mg/l)	0.00358
Converted $NOAEC_{external}$ (mg/l)	0.090
$NOAEC_{internal}$ (µg/l)	33
Corrected $NOAEC_{internal}$ (µg/l) = internal POD_{local}	17.6

3.1.3 Margin of exposure (MOE) for local effects of diacetyl

Based on equation 8 and Table 8.6 the MOE of diacetyl for local toxicity is calculated:

	Internal POD_{local} (µg/l)	Calculated exposure max. alveolar concentration		MOE	
		Low (µg/l)	High (µg/l)	Low	High
Local effect upon single exposure	167	0.6	1.3	278	128
Local effect upon repeated exposure	17.6	0.6	1.3	29.3	13.5

After single exposure, there is no risk for developing local lung toxicity when vaping e-liquids containing diacetyl in a concentration of 0.287 mg/ml. In the case this e-liquid with diacetyl is vaped repeatedly, for several months, the risk depends on whether the user is an average vaper or heavy vaper. However, if the e-liquid consumption of the user is rather low, then repeated exposure of diacetyl is not associated with a risk. However, high consumption of a diacetyl containing e-liquid results in a significant risk for local lung toxicity as the MOE < 25.

3.2 Systemic inhalation toxicity

3.2.1 Exposure assessment

The exposure assessment is also based on the highest amount of diacetyl found in e-liquids which was 287 µg/ml e-liquid (see Chapter VII).

Based on equation 9:

E-liquid concentration (mg/ml)	0.287*
Daily e-liquid consumption (ml)	5
Average body weight (kg)	60
Absorption (dead space volume) (%)	70
SED (mg/kg bw / day)	0.017

* Experimental data obtained in Chapter VII.

3.2.2 Point of Departure

For the POD_{sys}, the NOAEL reported by Colley et al. for a 90-day oral toxicity study in rats is used. At the highest dose which was 540 mg/kg/day, rats showed a decreased body weight gain, an increase in water consumption, increased blood leukocytes and an increase in relative weights of liver, kidney and adrenal and pituitary glands. There was also macroscopic and microscopic evidence of severe irritancy in both the glandular and non-glandular parts of the stomach. The NOAEL in this study was 90 mg/kg/day [14].

Critical endpoint	Macroscopic lesions of the stomach epithelium related to increases in liver, adrenal, and kidney weights
Study	Colley et al, 1969 [14].
Species	Sprague-Dawley rats
Exposure route	Oral
Doses tested (mg/kg /day)	0, 10, 30, 90, or 540
Duration of exposure (days)	90
NOAEL (mg/kg/day)	90

Based on Table 8.7

POD_{sys} = NOAEL (mg/kg bw/day)	90
Absorption	100%
Internal NOAEL (mg/kg bw/day)	90
Corrected NOAEL (mg/kg/day) = internal POD_{sys}	22.5

3.2.3 Margin of exposure (MOE) for the systemic effects of diacetyl

Based on Table 8.8, the MOE for systemic toxicity is calculated:

Internal POD_{sys} (mg/kg/day)	Calculated exposure SED (mg/kg bw / day)	MOE
22.5	0.017	1324

As the MOE ≥ 100, it can be concluded that there is no risk for systemic inhalation toxicity upon repeated exposure to diacetyl via vaping.

4 DISCUSSION

A risk assessment methodology for components inhaled through vaping was proposed earlier by the RIVM and was applied here for diacetyl in e-liquids. Risk assessments are an important part to evaluate the use of e-cigarettes and their impact on public health. Especially for the flavouring components in e-cigarettes, as these are substances for which in most cases toxicological data is already available. However, the main unknown usually is the exposure through vaping.

Therefore, in order to generate reliable exposure data some assumptions were made regarding the dynamics of e-cigarette use. Indeed, a model for intentional inhalation of substances was proposed. In addition, assumptions for the toxicokinetics were made such as the rate and amount of absorption and the rate of diffusion in the lungs. This methodology could be improved substantially if more measured data could be made available to fill up the assumptions that were made. The main uncertainties are situated within the dilution factors used for the exposure assessment. Also a conservative approach was used as the topography of e-cigarette use is not standardized nor uniform between users.

Also, the MOE has been determined using published NOAECs of a short term and a repeated inhalation study in rats and mice. More accurate would be to use human occupational exposure data. This data is currently only available for cases of occupational exposure in popcorn factories. Workers of these

factories were exposed to diacetyl among other substances and were linked to a clustered occurrence of bronchiolitis obliterans, a rare inflammatory disease leading to obstruction of bronchioles. However, as other substances are involved such as acetaldehyde, it is rather difficult to conclude that these clinical consequences are solely due to diacetyl.

Another limitation that needs to be taken into account in this case study is that the exposure assessment is based on the diacetyl concentrations that were measured in the e-liquids during the market survey, as explained in Chapter VII. It would, however, be more appropriate to determine the diacetyl concentration in the aerosol. In other studies, such as the one from Allen et al. [15], the concentration of diacetyl has been measured, but was expressed as mg/e-cigarette which makes it difficult to convert to the units of mg/ml as the volume of "an" e-cigarette is not known. In the earlier mentioned RIVM report [3] two e-liquids have been used for which diacetyl aerosol concentrations of 0.2 µg/ml and 0.23 µg/ml were measured. These e-liquids contained, however, a higher diacetyl concentration than the amount measured in our e-liquid sample, which makes their risk higher. On the other hand, also lower diacetyl concentrations than ours have been reported [8], [16]. It is clear that the e-liquid consumption is a critical factor in risk assessment as the MOE for local toxicity in case of repeated exposure is significantly different between low and high e-liquid consumption. Thus, there is an urgent need for reliable information on the e-liquid consumption for all e-cigarette devices. There should be an immediate follow up whenever new generations of e-cigarettes reach the EU market. Also, the topography of vaping is a critical factor that significantly contributes to the MOE. The higher the number and volume of puffs (Figure 8.4), the higher the alveolar concentration and thus the probability for the occurrence of local adverse health effect. This information could be retrieved from surveys among e-cigarette users to obtain a more realistic picture.

Overall, the risk assessment exercise shows that there is a health risk associated with the use of e-cigarettes containing diacetyl. Indeed, chronic exposure of the respiratory tract to diacetyl concentrations, as measured in our e-liquid samples, might result in local lung toxicity. Moreover, the damage pattern in the mice after chronic exposure seem to be comparable with the symptoms produced by bronchiolitis obliterans in humans [17]. Most e-cigarette users, however, tend to change e-liquid flavours and brands on a regular basis, thereby lowering the risk for adverse health effects since as such repeated exposure to the same component is limited.

Our risk assessment did not point to health effects with respect to local lung irritation when a single exposure to diacetyl was considered. Also no systemic toxicity could be observed after repeated exposure to diacetyl via inhalation.

5 CONCLUSION

Overall, this case study could show that a potential risk for local respiratory toxicity exists after chronic exposure to diacetyl via vaping. It supports the idea that the current EU regulatory framework for e-cigarette products should become more stringent and that positive and negative lists for flavours should be developed, comparable to those of fragrances in cosmetic products.

CHAPTER IX - IDENTIFICATION OF FLAVOURING SUBSTANCES OF GENOTOXIC CONCERN IN E-CIGARETTE REFILLS

As mentioned in the introduction, the toxicological profile of e-liquid components like nicotine, PG and G is relatively well established. However, there is still a need for more toxicological data on flavourings. While the flavourings diacetyl and acetylpropionyl have been discussed in more detail in Chapters V and VIII, we focus here on the identification of potentially genotoxic flavouring substances present in e-liquids. Genotoxicity has been associated with serious adverse human health effects including cancer. Consequently, genotoxicity testing plays a key role in the safety assessment of consumer products including e-cigarettes. With more than 7000 different flavoured e-liquids on the market, this poses a huge challenge not only from a practical but also from an economic and animal welfare point of view. Therefore, in this chapter, an animal-free screening strategy is proposed to prioritize e-liquid flavourings of the highest concern for human health from a genotoxic perspective. Potentially genotoxic flavouring substances are identified through a combination of analytical screening, *in silico* prediction tools and literature consultation. For a selection of flavourings, additional *in vitro* genotoxicity testing was performed. Both the bacterial reverse gene mutation test (also referred to as 'Ames test') and the *in vitro* micronucleus assay were applied, allowing to detect gene mutations and structural and numerical chromosome aberrations, respectively.

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1 INTRODUCTION

At present, the e-cigarette is the most popular alternative to tobacco smoking [18]. Unlike the traditional nicotine replacement therapy, e-cigarettes are available in many different flavours which is one of the main reasons for their popularity [19]. Today, more than 7000 different flavoured e-liquids are sold worldwide [20]. To obtain these flavours, synthetic chemicals, tobacco extracts or other herbal extracts are added to the e-liquids, collectively referred to as 'flavourings' [21].

Flavourings are also used in some conventional tobacco products. In the US, the tobacco industry uses more than 500 different additives, accounting for 10% of the total cigarette content, to improve the taste of tobacco cigarettes [22]. However, in Europe, the use of flavourings and other additives (vitamins, caffeine, certain ammonia compounds,…) in traditional tobacco cigarettes is banned as they

might further encourage the use of tobacco cigarettes and maintain the nicotine addiction of the user. A similar concern has been raised regarding the use of flavourings in e-cigarettes as the large variety of 'trendy' flavours makes e-cigarettes more attractive, especially amongst minors and young adults [23]. Consequently, e-cigarettes containing nicotine may initiate nicotine addiction and function as a gateway to tobacco cigarettes in this vulnerable group. Another concern of e-cigarette use relates to the potential toxicity following inhalation of flavourings. In tobacco cigarettes, the toxicity of additives is considered of minor significance compared to the toxicity induced by the tobacco-associated components [22]. However, when used in e-cigarettes, flavourings may be the main contributor to adverse human health effects. Many of the flavourings present in e-cigarettes are food grade or fragrances used in cosmetics. However, their toxicological profile is often poorly characterized, especially upon inhalatory exposure. Some flavourings commonly used in e-cigarettes, such as diacetyl and acetylpropionyl, are known to cause a local inflammatory lung disease, i.e. bronchiolitis obliterans, when repeatedly used [15]. Additionally, some specific strawberry flavourings used in e-cigarettes have been reported to induce significant toxicity *in vitro* such as a decrease in cell viability, metabolic activity and release of inflammatory mediators (cytokines) in H292 human bronchial epithelial cells [24]. Furthermore, flavourings present in e-liquids might undergo chemical reactions in the e-liquid mixtures or during the heating process, resulting in potential harmful reaction products such as flavorant–propylene glycol adducts and the formation of toxic aldehydes [25], [26].

While regulations for tobacco cigarettes mainly focus on reducing the appeal and the addictiveness, those for e-cigarettes are aimed at regulating the ingredients of the products themselves to assure consumer safety. To this extent, the EU Member States have revised the Tobacco Product Directive (2014/40/EU) (TPD) and adopted Article 20 herein that specifically relates to electronic nicotine delivery devices. In this Article 20, the minimum general requirements for e-liquid ingredients are included [27]. As a basic safety precaution, ingredients with Carcinogenic, Mutagenic or Reprotoxic (CMR) properties are banned in e-liquids.

Yet, another more general TPD requirement stipulates that 'flavourings, like other e-cigarette ingredients, are only allowed if they do not pose a risk to human health in heated or unheated form'. In this context, Girvalki et al. verified the health hazard statements defined by the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) for the most frequently found flavourings in e-liquids [28]. As such, 11 flavourings were identified to display hazards related to reproductive toxicity, organ toxicity upon single (benzyl acetate) or repeated exposure (banana oil), acute oral/dermal/inhalation toxicity (allyl hexanoate) and severe skin burns (phenol, 3,5-bis(1,1-

dimethylethyl)-) and eye damage (geraniol). It should, however, be noted that these are hazard statements and do not necessarily reflect a risk.

Furthermore, the TPD states that impurities in e-liquids are only allowed if they are technically unavoidable during manufacture and kept at trace levels. Hence, to minimize potential risks from contaminants such as volatile organic components (VOCs), the use of high purity flavouring ingredients is recommended [29].

Besides specific requirements for certain ingredients, the manufacturers of e-liquids are also obliged to notify their products before they are placed on the EU market (EU 2015/2183). In this notification, a list of ingredients should be provided with toxicological information on all ingredients [30]. However, the listing highly depends on the goodwill of the manufacturers. Some manufacturers are not eager to provide the required information, because of privacy and confidentiality issues. In the Decision (EU) 2015/2183, however, it is stated that ingredients present at a level below 0.1% in the final product formulation may be deemed confidential or a trade secret. Consequently, these ingredients are often described collectively in the notification by an umbrella term such as e.g. 'strawberry flavouring'. In most cases, the complete composition of the e-liquid thus remains unclear to the authorities (and even to the manufacturers), especially when natural extracts are used (tobacco extracts, essential oils, herbal extracts, or non-chemically synthetized flavourings), as their composition is not always known and may vary from batch to batch depending on biological and geographical origins [31]. It is thus highly likely that e-liquids contain substances with unknown toxicological properties or known toxic components that exceed certain limits. In those cases, the use of e-liquids might cause adverse human health effects.

Although their presence is legally not allowed, previous studies have shown that (potential) CMR substances occur in certain e-liquids. More specifically, VOCs such as benzene, toluene, etc. have been found to be present as residual solvents in tobacco extracts [16], [32]. Also, for many of the flavourings in e-liquids, toxicological data are limited or even absent and their CMR properties remain thus unknown. Ideally, full characterization of all ingredients used in e-liquids is a first important requisite. Next, the toxicological properties of the ingredients and/or the whole e-liquid need to be identified to be able to assess their safety and evaluate possible risks associated with e-cigarette usage. Considering that there are more than 7000 different flavoured e-liquids on the market, we developed a prioritization strategy to identify potentially genotoxic substances used as e-liquid flavouring. The strategy followed only uses non-animal methods and is based on a similar approach that has recently been applied to identify genotoxic compounds used in printed paper and board food contact materials [33]. As such, the prioritization strategy consists of four steps: (i) Identification of the chemical

substances present in the e-liquids via GC-MS screening; (ii) Prediction of the genotoxic potential of the substances using two complementary (quantitative) structure-activity relationship (or (Q)SAR) *in silico* models; (iii) Collection of existing *in vitro* and *in vivo* genotoxicity data from public literature sources; and (iv) *In vitro* genotoxicity testing on a selection of commercially available flavourings. Based on all collected information, flavourings of high concern were identified.

2 MATERIALS AND METHOD

2.1 Chemicals

The positive control substances for the genotoxicity assays i.e. benzo[a]pyrene (BaP), sodium azide, 2-aminoanthrancene and 4-nitroquinoline 1-oxide (4-NQO) and dimethylsulfoxide (DMSO), were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). An overview of the substances tested *in vitro* and reference standards used for chemical confirmation is presented in Table 9.1.

Table 9.1: Overview of the test compounds included in the *in vitro* genotoxicity study.

Name	CAS number	Provider	Purity (%)
safrole	94-59-7	Sigma Alrich	99.6
estragole	140-67-0	Sigma Alrich	99.3
furylmethylketon	1192-62-7	Sigma Alrich	99.8
transhexenal	6728-26-3	Sigma Alrich	98.4
2,5-dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	Sigma Alrich	98.0
β-phellandrene	555-10-2	TRC Chemicals	96.0
isoledene	95910-36-4	Sigma Aldrich	98.5
2,3-butanedione	431-03-8	Sigma Aldrich	99.5
2,3-pentanedione	600-14-6	Sigma Aldrich	97.1

2.2 Screening of e-liquid samples using GC-MS

A total of 129 e-liquids representative for different flavour categories [34], present on the Belgian market, were collected and screened analytically. The samples were either obtained upon inspections of different vaping shops in Belgium or were seized postal packages ordered by individuals through the internet between 2016 and 2018. The identification of the components present in the e-liquids was done by using the National Institute of Standards and Technology (NIST) research library. A library spectral match quality of 85% was considered as a positive identification. A peak area threshold of

0.1% of the main peak was set in order to exclude contaminations from carry-over in the GC-capillary column (polysiloxanes). Two different GC-MS screening methods were used to cover the wide variability of volatility among the different compounds.

2.2.1 Method A: high volatiles

The screening was performed on an Agilent 6890 N gas chromatograph coupled to an Agilent 5973N single quadrupole mass spectrometer and equipped with a G188A static headspace sampler (Agilent Technologies, Palo Alto, USA). The samples were diluted by dissolving 1 g e-liquid sample in 10 ml water of which 300 µl was transferred to a 10 ml sealed vial, placed in the autosampler oven to be heated and agitated in order to generate a gas phase. The incubation temperature was maintained at 85°C with an equilibration time of 15min. The injector port was kept at 16 °C, in split injection mode (split ratio 15:1), while the temperatures of the headspace loop and the transfer line were maintained at 100 and 120°C, respectively. The components were separated on a VF-5 ms (5% phenyl-95% methylpolysiloxane) capillary column of 60 m with Ø 0.25 mm and film thickness of 0.25 µm and an integrated guard column of 10 m (#CP9013, Factor four, Agilent, California, USA). Helium carrier gas was used at a constant flow of 1.0 ml/min. The initial oven temperature of 45°C was maintained for 10 min, followed by a temperature ramp of 40°C/min to a final temperature of 250°C. The total run time was 18 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV. Temperatures of the ion source, the quadrupole, and the interface were set at 230, 150 and 280°C, respectively. The identification was performed in full scan mode from 25 to 400 m/z.

2.2.2 Methods B: semi-volatiles

The second screening method was applied to detect the semi-volatile components in e-liquids. Thus, the incubation temperature was maintained at 145°C with an equilibration time of 15 min to obtain full evaporation mode. To minimize the interference of the matrix components propylene glycol and glycerol, a liquid-liquid extraction was applied as sample preparation step. The components were extracted with hexane followed by separation through a “freeze-and-pour” technique. For each sample extraction, 0.3 g of the e-liquid was weighted in a glass vial of 20 ml, mixed with 3 ml hexane and covered with a Teflon seal. During the first extraction step, vials were vortexed for 10 s and then sonicated for 3 min at 50°C. Afterwards, vials were transferred to a cooling bath of -78°C (dry ice dissolved in acetone) for 2 min, followed by 1 min centrifugation at 860 g. Three quarters of the supernatants was transferred to a glass vial. These extraction steps were repeated again by adding another 3 ml of hexane. After extraction, 300 µl of the extracted solution was transferred to a headspace vial of 10 ml. The GC-MS conditions were similar to those of the method for high volatiles,

except for the temperature gradient. The temperature gradient started at 65°C (held for 3 min) and raised with 5°C/min to reach 90°C. The temperature gradient continued at 20°C/min, until 185°C, followed by another fast decrease in temperature to 100°C by 30°C/min, that finally increased with 35°C/min until 290°C (held for 3 min). The total runtime of the method was 24 min.

2.3 *In silico* prediction of genotoxicity

The genotoxic potential of the compounds identified through the analytical screening of the e-liquids was investigated *in silico* using two complementary (quantitative) structure-activity relationship ((Q)SAR) models (Derek Nexus™ and Sarah Nexus™). . The combination of the Derek and Sarah Nexus models is applied in the current study. Nevertheless other (Q)SAR models are available. However, it is generally recommended to use a combination of rule-based SAR (Derek NexusTM) and statistics-based QSAR (Sarah NexusTM) tools to assess Ames mutagenicity. For example, the ICH-M7 guideline for the assessment and control of mutagenic impurities in pharmaceuticals, specifically requires to combine a SAR with a QSAR model as each type of model has its (dis)advantages as reviewed by Honma et al. [35]. Based on the Chemical Abstract Service (CAS) number, the simplified molecular input line entry systems (SMILES) of the substances were extracted via PubChem (National Institutes of Health) [36] and ChemSpider (Royal Society of Chemistry) [37]. The SMILES were entered into the two different computational systems (i.e. Sarah Nexus 1.1.19 and Derek Nexus version 5.0.2). Predictions were only made for the endpoint '*in vitro* bacterial mutagenicity'. For Derek, substances were considered positive if the prediction outcome for this endpoint was '*certain*', '*probable*', '*plausible*' or '*equivocal*' whereas for Sarah, substances were considered positive if the prediction outcome was '*positive*' or '*equivocal*'.

2.4 Genotoxicity data collected from EU databases

For the flavouring components with a positive prediction outcome for '*in vitro* bacterial mutagenicity' in at least one of both (Q)SAR models, genotoxicity data was collected from previous safety evaluations by European Authorities from different regulatory domains using the strategy previously proposed by Van Bossuyt et al [33]. In the first step, the genotoxic potential of the compound was verified in the harmonized classification according to the CLP regulation [38]. If there was no harmonized CLP classification available, genotoxicity data were collected from evaluations by EU authorities i.e. Opinions issued by the European Food Safety Authority (EFSA) via the Open Food Tox database [39] and by the Scientific Committee on Consumer Safety (SCCS). In case no evaluation of the genotoxic potential was available in these Opinions, the European Chemicals Agency (ECHA) database was consulted [40]. The ECHA database has been constructed under the framework of the

Registration, Evaluation, Authorization And Restriction Of Chemicals (REACH) regulation, which establishes procedures for collecting and assessing hazards of substances [41]. Chemical manufacturers need to register their substances (if manufactured or imported in the quantity of 1 ton or more per year) and provide amongst others information on toxicological data. The approach described by Mertens et al. was used to retrieve genotoxicity data from the ECHA database [42].

2.5 *In vitro* genotoxicity testing

Components with a positive prediction in at least one of the two (Q)SAR models and for which no *in vitro* genotoxicity data was found and that were commercially available, were tested *in vitro* using a battery of two tests i.e. the Ames test and the *in vitro* micronucleus test. The former detects gene mutations [43], whereas the latter picks up structural and numerical chromosomal aberrations [44].

2.5.1 Ames test

The test was performed according to the OECD Guideline for testing of chemicals, Test No. 471: Bacterial reverse mutation test [45] with slight modifications. Normally the OECD recommends to use five bacterial tester strains to cover the full range of mutagenicity. In the present screening study, only the strains *Salmonella typhimurium* TA98 and TA100 were used. TA100 is the most sensitive strain, detecting 83% of the mutagens whereas TA98 detects 67%. When used in combination, TA98 and TA100 are able to pick up approximately 93% of all 224 mutagens tested by the National Toxicology Program [46].

Salmonella typhimurium bacteria (TA98 or TA100) (Moltox, Boone, USA) were grown overnight and 100 µl of the bacterial suspension was mixed with 100 µl of the test solution, 500 µl sodium phosphate buffer pH 7.4, and 2 ml overlay agar enriched with a histidine-biotine solution. To test the substance in its metabolized form, the buffer was replaced by a 5% S9 metabolization mix (prepared from lyophilized rat liver S9 mixed with nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system – both from Moltox). The resulting mixture was poured onto a minimal glucose agar plate (E&O Laboratories Ltd., Bonnybridge, United Kingdom) and incubated for 48 hours at 37°C (Binder, Tuttlingen, Germany). Triplicate plates were poured for each test condition. All substances were tested in at least five concentrations as prescribed by OECD test guideline 471 (OECD, 1997). Before the start of the experiment, a dilution range of the test compound was made. Dimethylsulfoxide (DMSO) was used as a solvent for the test compounds. Positive, negative and solvent control plates were prepared in parallel with the test substance plates. As positive controls, 4-nitroquinoline-N-oxide (4NQO; 2µg/ml; TA98 without S9), sodium azide (20 µg/ml; TA100 without S9) and 2-aminoanthracene (10 µg/ml; TA98 and TA100 with S9) were used. After 48 hours, the amount

of revertant colonies was counted and compared to the amount of revertants present in the solvent control. A compound was considered mutagenic *in vitro* in case the amount of revertants had doubled compared to the solvent control. The effect also needed to be concentration-dependent.

2.5.2 *In vitro* micronucleus assay

The *in vitro* micronucleus test was carried out following the OECD guideline 487, with some modifications [47]. CHO-K1 cells were seeded at a density of 2.0×10^5 cell/ml (with S9) or 1.0×10^5 cell/ml (without S9) and exposed to five different concentrations of the test substance in the absence (24 h) or presence of S9 fraction (4 h). The test concentrations were selected based on the results obtained in cytotoxicity assays. The PBS medium with DMSO was used as negative control; 15 µg/ml methyl methanesulphonate (MMS) (without S9 fraction) and 25 µg/ml benzo(a)pyrene (BaP) (with S9 fraction) as positive controls. After exposure to the test compound, cells were incubated for 21h with cythochalasin B (Cyt-B) (3 µg/ml) to block cytokinesis and to obtain binucleated cells. Afterwards, cells received a hypotonic treatment with KCl followed by fixation. Next, cells were smeared onto clean microscopic glass slides. The slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and evaluated for the presence of micronucleated binuclear cells using a Zeiss Axiovert 40 microscope with MetaFer4 version 3.13.0 using MNScoreX software for analysis of micronuclei. In total, two slides were analyzed per test condition and 1100 cells were scored per slide resulting in at least 2000 evaluated cells per test condition.

After analysis for the presence of micronuclei, cells were stained with acridine orange (AO) (33.3 µg/ml) to evaluate cytotoxicity. Slides were rinsed with Sörensen buffer to eliminate excess of staining solution and for approximately five hundred cells per test condition, the number of mono-, bi- and multinuclear cells was determined manually. The level of cytotoxicity was evaluated by calculating the cytokinesis-block proliferations index (CBPI) which is defined as the ratio of:

$$\frac{mononuclears + 2 \times binuclears + 3 \times trinuclears + 4 \times tetra\ (and\ higher) nuclears}{total\ viable\ cells}$$

Results were summarized and analysed with GraphPad Prism version 7.01. A Fisher's exact test was performed in GraphPad to evaluate whether there was a statistically significant difference between a test condition and the negative control ($p < 0.05$). The chi-square test was used to evaluate whether the test compound induced a dose-dependent effect ($p < 0.05$).

3 RESULTS

An overview of the results obtained in the different steps of the prioritization strategy to identity genotoxic flavourings in e-liquids is given in Figure 9.1.

3.1 Screening of e-liquid samples using GC-MS

After screening 129 e-liquids, 807 individual chemical substances were identified including nicotine, nicotine-impurities, VOCs impurities, additives (diacetin) and flavouring components (incl. synthetic, components from essential oils or other herbal extracts). The NIST provided a CAS number for each identified component which was also used to retrieve the SMILES formula in PubChem or ChemSpider [36], [37].

3.2 Genotoxicity prediction using *in silico* tools

Out of the 807 components analysed, only 81 showed a positive prediction outcome for *in vitro* bacterial mutagenicity in at least one of the two (Q)SAR models. Consequently, 90% of the screened components were concluded to be negative for *in vitro* bacterial mutagenicity in the applied *in silico* models as negative predictions for *in vitro* bacterial mutagenicity were produced by both models. However, of these negative predictions, eight were labeled as containing “misclassified features” and six “unclassified features”. When a query does not fire an alert for mutagenicity *in vitro* in Derek Nexus, the compound is compared to a reference set of Ames test data. In the case a chemical fragment, or “feature”, is unclassified, it is present in the query but not the reference set, whereas a misclassified feature is found in a reference set compound which is positive in the Ames test, but not defined as a structural alert, so the relationship between these features and mutagenic activity may be coincidental (or contributory). Since the negative predictivity remains high for both, these can be regarded as a negative prediction that are flagged for expert review [48]. In our strategy, we did not include an elaborate expert reviewing process and these were thus accepted to be negative.

Overall, only 8 components were predicted positive in both *in silico* tools. Interestingly, most positive predictions for *in vitro* bacterial mutagenicity were obtained with the quantitative prediction model Sarah Nexus™. Yet, the two *in silico* tools used are based on a different methodology and different training sets which might explain the significant difference in the number of positive prediction outcomes.

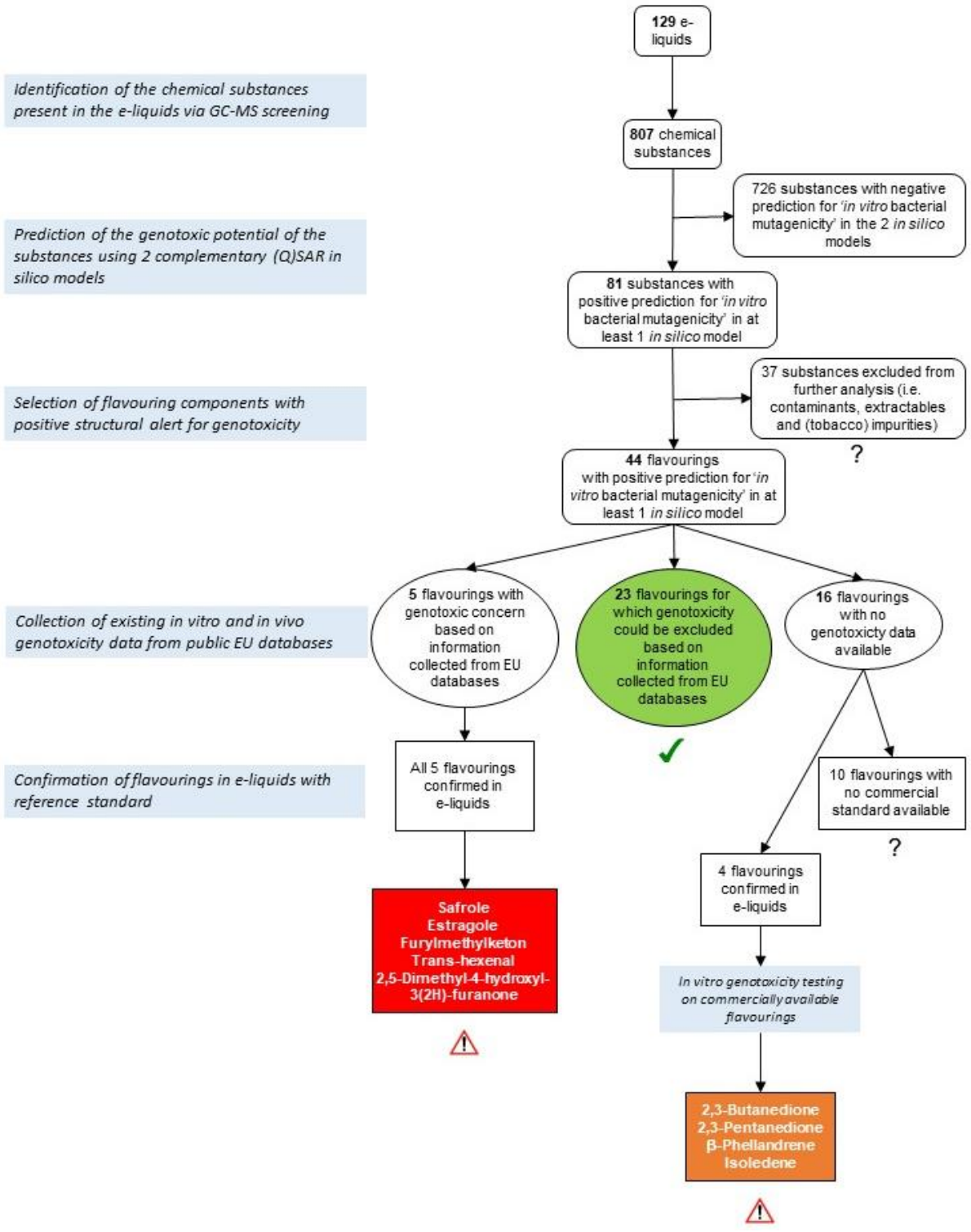


Figure 9.1: *Summary of the results obtained with prioritization strategy to identity genotoxic flavourings in e-liquids.*

3.3 Genotoxicity data collected from EU databases

As the present study focused only on flavourings, contaminants/extractables and (tobacco) impurities that can also be present in e-liquids were first excluded before starting the data collection. However, manufacturers should be aware of the presence of these types of compounds and regulators need to address them explicitly in future regulatory amendments. In total, 37 compounds belonging to the group of 'contaminants/extractables and (tobacco) impurities' were identified among the 81 substances with a structural alert for mutagenicity in at least one of the two (Q)SAR models. Consequently, 44 out of the 81 substances with a positive prediction outcome for *in vitro* bacterial mutagenicity in at least one of the two (Q)SAR models were flavourings. Probably there are more 'contaminants/extractables and (tobacco) impurities' among the 807 that have been analytically identified. However, selection of the flavourings was only done after applying the *in silico* models as looking into all 807 components would have been too time-consuming.

Based on the collection of the information from EU databases for the 44 flavourings, five compounds with a concern for genotoxicity could be identified and for 23 flavourings genotoxicity could be excluded. However, for 16 flavourings no genotoxicity data was available according to the consulted literature and thus genotoxic potential could not be excluded (Table 9.2.).

Flavourings of high genotoxic concern

For 1 out of the 44 flavouring substances, a harmonized CLP classification for mutagenicity has been established. Indeed, **safrole** has been classified as a Mutagen category 2 and a Carcinogen category 1B. According to an opinion of the Scientific Committee on Food [49], safrole may not be used as a flavour in food due to these CMR classifications, but it is often found in essential oils. Essential oils containing safrole should not be used at a level such that the total concentration of safrole exceeds 0.01% in consumer products. Examples of essential oils with a high safrole content are Sassafras oil, Ocotea Cymbarum oil and certain qualities of Camphor oils. These recommendations are based on the conclusions of the Scientific Committee on Cosmetology of the EEC on safrole [50].

For another substance, **estragole**, the (*in vivo*) genotoxicity has been generally acknowledged in a previous evaluation by the European Medicine Agency (EMA) [51]. Estragole is mostly found in tobacco flavours to add a herbal anise aroma, but it can also be part of various natural extracts. A large number of plants and their preparations have been reported to contain estragole, sometimes in very high amounts such as *Foeniculum vulgare Mill.* (both fruit and essential oil) and Pimpinella anisum L. (fruit).

2,5-Dimethyl-4-hydroxy-3(2H)-furanone has previously been evaluated by EFSA and was considered to be genotoxic *in vivo*. 2,5-dimethyl-4-hydroxy-3(2H)-furanone is also called strawberry furanone because it is used to add a strawberry aroma to food. Several *in vitro* and *in vivo* genotoxicity studies indicate that the substance induces mutagenic responses [52]. In-depth investigation shows that the observed positive results are due to the production of reactive oxygen species, potentiated by the presence of metals in the cell medium. The resulting DNA damage is only observed once the cell antioxidant capacity has been exhausted. EFSA stated that this effect is unlikely to occur at the low levels used for flavourings in foods. Yet, available data for this substance clearly illustrates that a separate risk assessment is needed for exposure to these concentrations through inhalation of e-cigarettes.

For two compounds, **trans-hexenal** and **2-furylmethylketon,** an evaluation was done by EFSA, but no conclusion on the *in vivo* genotoxic potential of the compounds could be made and therefore further data was requested [53], [54]. 2-Furylmethylketon is used as a food flavouring and fragrance and can also be found in tobacco. The EFSA concluded that the genotoxic potential of furylmethylketon could not be excluded. Based on the available experimental information the substance may give rise to DNA damage, possibly resulting in chromosomal aberrations rather than gene mutations [53]. Trans-hexenal was found in e-liquids with a green fruit flavor. Also for this compound, EFSA could not exclude a genotoxic concern. Both gene mutations in *Salmonella typhimurium* TA100, and chromosomal aberrations in mammalian cells were observed *in vitro.* However, available experimental data from animals did not show an induction of gene mutations by trans-hexenal [54]. In contrast, the available data were insufficient to assess the clastogenic potential of this compound at the first site of contact and in the liver *in vivo*. Therefore, the genotoxicity concern could not be ruled out by EFSA and additional information was required.

The presence of the five components mentioned with a concern for genotoxicity in the initial e-liquids was confirmed with reference standards used in GC-MS.

Table 9.2: Conclusion of the EU evaluations for genetic toxicity of the components with a positive *in silico* prediction for *in vitro* bacterial mutagenicity. Results are expressed as (+) positive, (-) negative or (±) inconclusive if genotoxicity could not be excluded and additional information is requested. European Medicine Agency (EMA), European Food and Safety Agency (EFSA).

Name	CAS	EU evaluation		CONCLUSION
estragole	140-67-0	EMA		+
safrole	94-59-7	Harmonized CLP		+
2,5-dimethyl-4-hydroxy-3(2H)-furanone	3658-77-3	EFSA		+
2-hexenal, (E)-	6728-26-3	EFSA		±
ethanone, 1-(2-furanyl)-	1192-62-7	EFSA		±
1,3-cyclohexadiene-1-carboxaldehyde, 2,6,6-trimethyl-	116-26-7	EFSA		-
1-hexen-3-one	1629-60-3	EFSA	based on read across data	-
2,4,6-octatriene, 2,6-dimethyl-	673-84-7	EFSA	based on read across data	-
2,4,6-octatriene, 2,6-dimethyl-, (E,Z)-	7216-56-0	EFSA	based on read across data	-
2-buten-1-one, 1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-, (E)-	23696-85-7	EFSA	based on read across data	-
2-furancarboxaldehyde, 5-methyl-	620-02-0	EFSA		-
2-propenal, 3-(2-methoxyphenyl)-	1504-74-1	EFSA	based on read across data	-
α-phellandrene	99-83-2	EFSA	based on read across data	-
benzaldehyde	100-52-7	EFSA		-
benzaldehyde, 2-methoxy-	135-02-4	EFSA	based on read across data	-
benzaldehyde, 4-methoxy-	123-11-5	EFSA		-
benzene, 1,1'-[oxybis(methylene)]bis-	103-50-4	EFSA		-
benzene, 1,4-dimethoxy-	150-78-7	EFSA		-
cinnamaldehyde, (E)-	14371-10-9	EFSA		-
ethanone, 1-(3-pyridinyl)-	350-03-8	EFSA	based on read across data	-
furfural	98-01-1	EFSA		-
γ-terpinene	99-85-4	EFSA		-
maltol	118-71-8	EFSA		-
phenol, 2-methoxy-	90-05-1	EFSA		-
piperidine	110-89-4	EFSA	based on read across data	-
piperonal	120-57-0	EFSA		-
pyridine, 3-ethyl-	536-78-7	EFSA		-
thiazole, 4-methyl-2-(1-methylethyl)-	15679-13-7	EFSA	based on read across data	-

Flavourings for which genotoxic concern could be excluded

In contrast, for 23 of the flavourings with a positive prediction for *in vitro* bacterial mutagenicity in at least one of the (Q)SAR models, a genotoxic concern could be excluded based on the information collected from EU databases (Table 9.2). These included components for which results were negative either in all *in vitro* tests or in the *in vivo* follow-up genotoxicity tests. For 10 of those components, the genotoxic concern was excluded based on read-across with genotoxicity data from substances with comparable structures. As these 23 are not considered to be genotoxic, no additional analysis was performed to confirm their presence in the e-liquids.

Flavourings for which more genotoxity data is needed

For the remaining 16 components (Table 9.3), no genotoxicity data was available in the consulted literature. As these components are of potential concern, confirmation of the presence of these components in the e-liquid was needed. However, this required the (commercial) availability of a reference standard which was not the case for 10 out of the 16 components. This can be explained by the fact that most of these components are tobacco or natural extracts. For 4 out of the 6 substances for which a standard was commercially available, their presence in the e-liquid could be confirmed with GC-MS using full scan mode. The other 2 substances were not detected in the e-liquid when using a reference standard-based methodology. The absence of these compounds in e-liquid is probably due to a mismatch with the NIST-library. As these two components were not present, they should not be further explored.

3.4 *In vitro* genotoxicity testing

Next, the Ames test and the *in vitro* micronucleus test were performed for the four commercially available flavourings with a positive prediction outcome in at least one of both two (Q)SAR models and for which genotoxicity data is lacking. These components included β-phellandrene, isoledene, 2,3-pentadione and 2,3-butanedione. An overview of the *in vitro* genotoxicity test results is given in Table 9.4.

Table 9.3: Overview of the substances with a positive prediction for *in vitro* bacterial mutagenicity and for which no information on genetic toxicity was available from EU authorities.

Name	CAS	Commercially available	Confirmed in e-liquid	CATEGORY
Potentially high concern				
β-phellandrene	555-10-2	YES	YES	flavour and fragrance use
isoledene	95910-36-4	YES	YES	natural extract
2,3-butanedione	431-03-8	YES	YES	flavour and fragrance use
2,3-pentanedione	600-14-6	YES	YES	flavour and fragrance use
Needs confirmation				
naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis)-	483-77-2	YES	NOT TESTED	natural extract
α -calacorene	21391-99-1	YES	NOT TESTED	natural extract
1,3,5-cycloheptatriene, 1-methoxy-	1728-32-1	YES	NOT TESTED	natural extract
α-methyl- α -[4-methyl-3-pentenyl]oxiranemethanol	1000132-13-0	NO	NOT TESTED	natural extract
1,3-dioxane, 2-methyl-	626-68-6	NO	NOT TESTED	natural extract
1H-azepin-1-amine, N-ethylidenehexahydro-	75268-01-8	NO	NOT TESTED	natural extract
bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	28634-89-1	NO	NOT TESTED	natural extract
cyclohexene, 4-methylene-1-(1-methylethyl)-	99-84-3	NO	NOT TESTED	natural extract
naphthalene, 1,2-dihydro-2,5,8-trimethyl-	30316-23-5	NO	NOT TESTED	natural extract
cyclohex-3-enecarboxaldehyde, 2,4,6-trimethyl-, oxime	1000294-92-2	NO	NOT TESTED	flavour
Not detected in e-liquid samples				
p-methylbenzyl acetate	2216-45-7	YES	NO	flavour and fragrance use
2H-Pyran-2-one, 4-hydroxy-3,6-dimethyl-	5192-62-1	YES	NO	flavour use

Table 9.4: Overview of the results in the Ames test (TA98 and TA100 strains) and the *in vitro* micronucleus test in presence and in absence of an S9 metabolisation system (S9) for commercially available substances with a positive prediction outcome in at least one of the two (Q)SAR models and for which genotoxicity was not previously published. Results are indicated as (+) positive or (-) negative.

	Ames test				***In vitro* micronucleus assay**	
	TA98		**TA100**			
	-S9	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
β-phellandrene	-	-	-	-	-	+*
isoledene	+	+	+	+	-	+
2,3-pentanedione	+	+	-	-	+	+
2,3-butanedione	+*	+	+*	+	-	+

** Only at the highest concentration tested*

3.4.1 Ames test

β-Phellandrene was first tested in *Salmonella typhimurium* TA100 in concentrations up to 1mg/ml both in the absence and presence of S9 metabolic fraction. No increase in the number of revertants compared to the solvent control was observed for any of the test conditions. However, cytotoxicity was present at concentrations above 0.03 mg/ml. For this reason, lower concentrations (0.5 to 50 µg/ml) were tested in the *Salmonella typhimurium* TA98 strain. The background layer was intact for all the concentrations tested and no mutagenic effect could be observed in the absence nor in the presence of S9 metabolic fraction. Consequently, β-phellandrene is considered not mutagenic in TA98 and TA100 with and without S9 metabolic activation (Figure 9.2A). A recent study reported that β-phellandrene induced a significant increase in the number of revertants compared to the solvent control in both strain TA98 and TA100 in the absence and presence of S9 metabolic fractions [55]. However, important study details (e.g. purity of the compound, solvent used,…) are lacking to allow adequate comparison of the outcome of this study with our results.

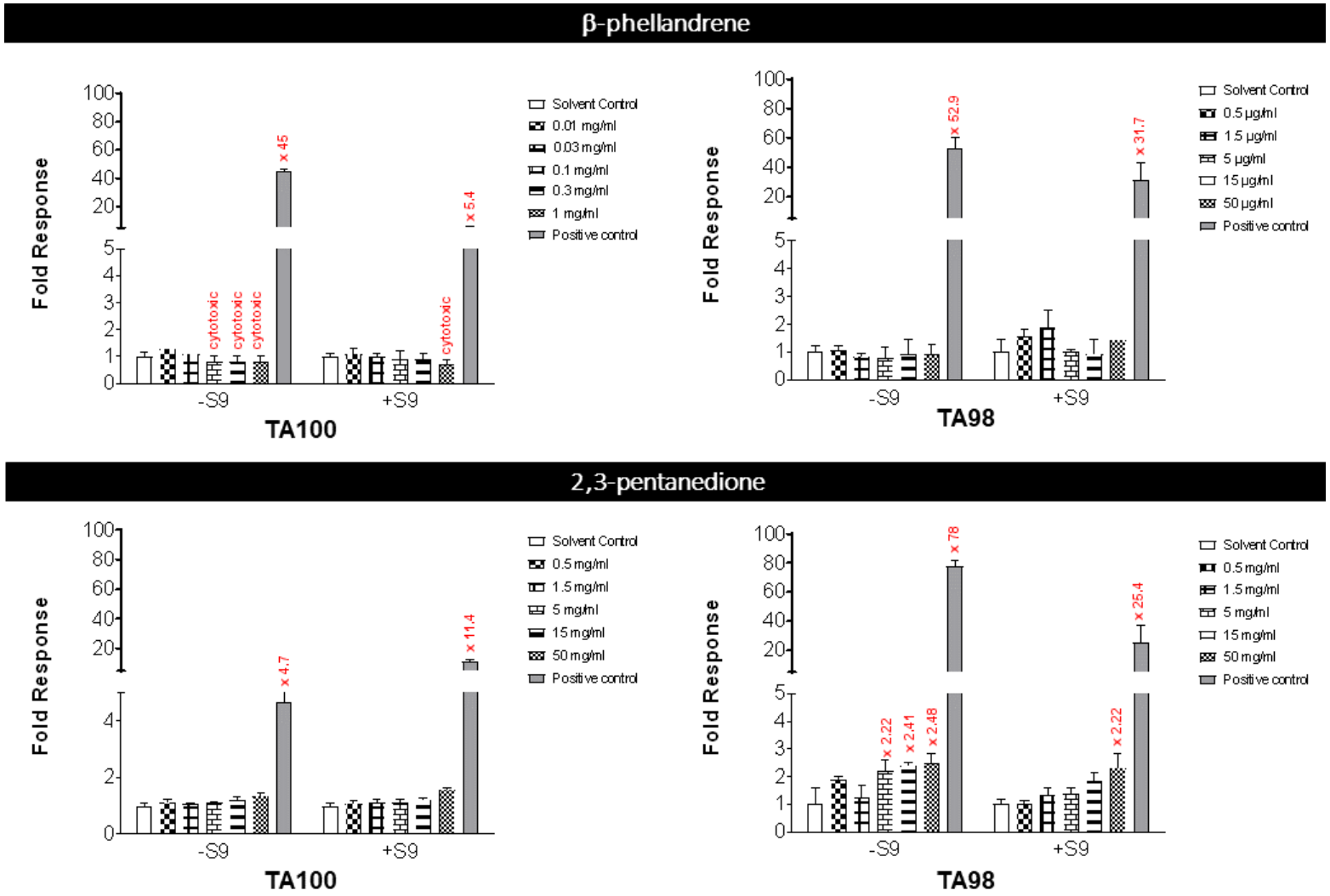


Figure 9.2A: *Ames test results, with and without S9, of investigated substances with positive prediction in silico. Values are expressed as the mean ± SD of revertant colonies counted in triplicate plates for each tested strain (TA100 and TA98) *Statistically significant compared to solvent control (p<0.05).*

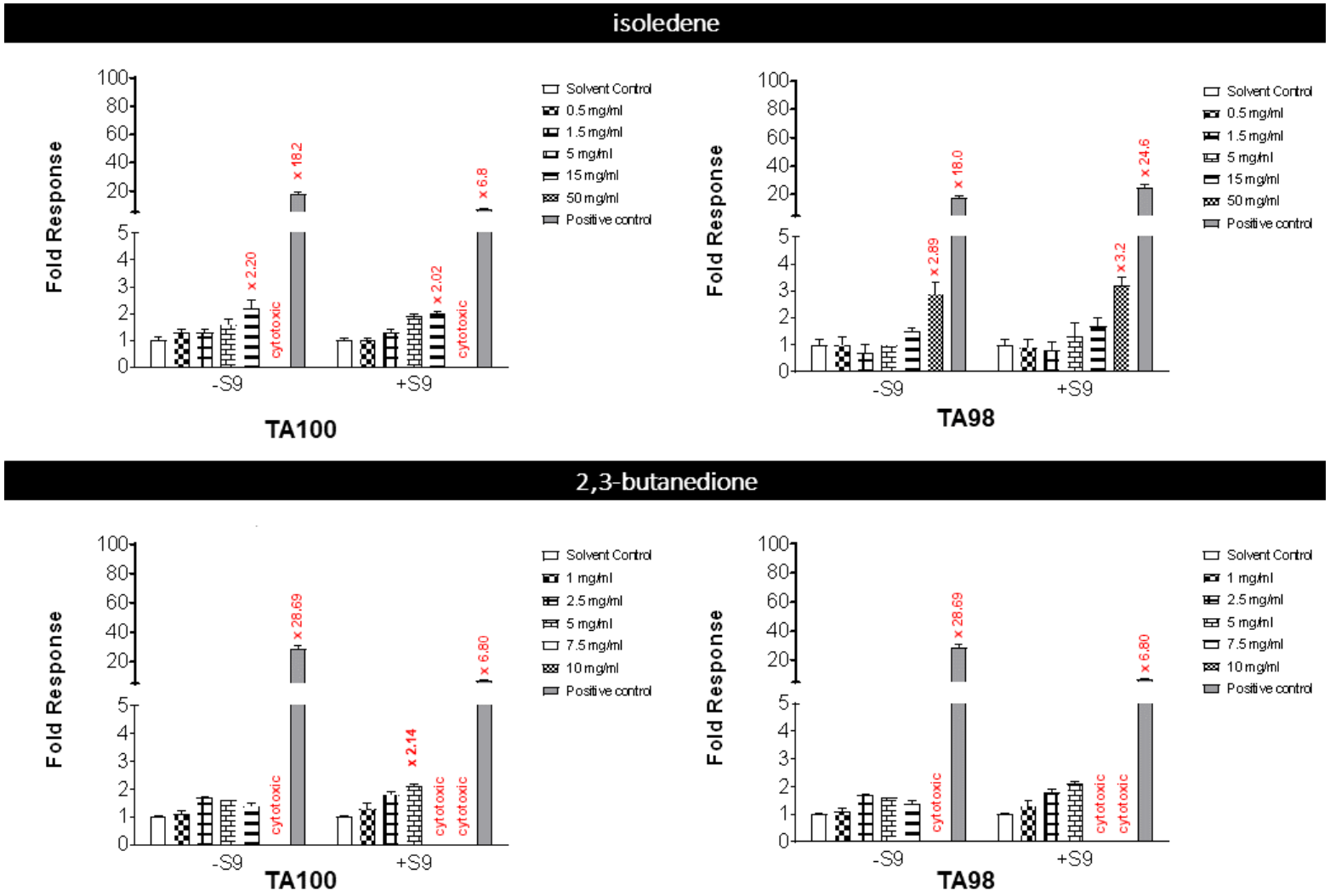


Figure 9.2B: *Ames test results, with and without S9, of investigated substances with positive prediction in silico. Values are expressed as the mean ± SD of revertant colonies counted in triplicate plates for each tested strain (TA100 and TA98) *Statistically significant compared to solvent control ($p<0.05$).*

2,3-Pentanedione did not induce a mutagenic effect in TA100 with or without S9 metabolic fraction (Figure 9.2A). In TA98, a concentration-dependent increase in the number of revertant colonies was observed which was more than double compared to the controls, starting from 5 mg/ml in the absence of S9 (Figure 9.2A). A concentration-dependent effect was also present with a metabolic activation system, although a doubling was only observed at 50 mg/ml. A uniform background layer was present at all tested concentrations indicating that the compound was not toxic to the bacteria. Based on these results, 2,3-pentanedione is considered to have a mutagenic capacity *in vitro*. Previous Ames tests did not, however, indicate mutagenicity for 2,3-pentanedione. For example, Aeschbacher et al. reported no mutagenic effects in the TA100 strain and in TA98 at concentrations ranging from 0.009 mg/ml to 900 mg/ml [56]. Florin et al. only tested 3 µmol/plate in TA100 (with and without S9) which also did not induce a mutagenic effect [57].

In TA100, **isoledene** induced a concentration-dependent increase in the number of revertant colonies which was more than the double the number of revertants present in the solvent control at 15 mg/ml (Figure 9.2B), both with and without metabolic activation. At the highest concentration (i.e. 50 mg/ml), cytotoxicity was observed. In TA98, a duplicate of the number of colonies was observed at 50 mg/ml with a concentration-dependent increase in the range of 5 mg/ml – 50 mg/ml. Based on these results, isoledene is considered to be mutagenic *in vitro*.

2,3-Butanedione was tested up to 10 mg/ml in the TA100 strain, but cytotoxicity was only observed at the two highest concentrations in the absence and presence of S9 metabolic fraction. At the lower concentrations, there was a clear concentration-dependent increase in the number of revertant colonies compared to the solvent control, both with and without S9 metabolic system. The number of revertant colonies was twice the number of the solvent control at 5 mg/ml with the S9 metabolic system. Therefore, 2,3-butanedione is considered to be mutagenic *in vitro* in TA100, both in the presence and absence of metabolic activation (Figure 9.2B). In TA98, 2,3-butanedione was slightly positive with and without metabolic activation at 2.5 and 5 mg/ml. At higher concentrations, significant cytotoxicity was observed, which was reflected by a decrease in the number of revertants. Aeschbacher et al. also reported a positive effect for 2,3-butanedione in TA102 (0.17 µg – 17.2 mg/plate), although in their study the compound was negative in both the TA98 and TA100 strains [56].

3.4.2 *In vitro* micronucleus test

In the *in vitro* micronucleus test without S9, no increase in the number of micronuclei in binucleated cells was observed with **β-phellandrene** in concentrations up to 0.68 mg/ml (Figure 9.3A). In the

presence of S9, a slight but statistically significant increase in the number of micronuclei was present, but only at the highest concentration tested, i.e. 2.72 mg/ml. Limited cytotoxicity of 5% was observed under this condition.

In the first experiment in the presence of S9, **isoledene** was tested up to a concentration of 1000 µg/ml. However, except for 250 µg/ml, cytotoxicity was observed in all conditions. At 250 µg/ml, an increase in the number of micronuclei in binucleated cells was present. Therefore, the test was repeated with lower concentrations of isoledene. An increased induction of micronuclei could also be seen at a concentration of 20 µg/ml (Figure 9.3A). In the absence of the metabolic activation system, no effect on the number of micronuclei was observed whereas cytotoxicity was present at a concentration of 100 µg/ml. The results of the present study indicate that isoledene causes an increase in the number of micronuclei in the presence of S9 metabolic fraction.

2,3-Pentanedione was evaluated in the *in vitro* micronucleus test at concentrations up to 50 µg/ml (without S9) or 200 µg/ml (with S9) (Figure 9.3B). A dose-dependent effect in the number of micronuclei was observed starting at 5 µg/ml without S9 and at 50 µg/ml with S9. However, in the presence of S9, the highest concentration tested induced no increase in the number of micronuclei because of cytotoxicity. In the absence of S9, cytotoxicity was observed at 50 µg/ml. The results of the present study indicate that 2,3-pentanedione induces chromosome damage in CHO-K1 cells.

In the first experiment in the absence of S9 metabolic fraction, **2,3-butanedione** was tested at concentrations between 10 µg/ml and 100 µg/ml. However, cytotoxicity was already observed at 25µg/ml. At the lower concentrations, 2,3-butanedione did not induce an increase in micronucleus formation. The test was repeated with concentrations ranging from 5 µg/ml and 20 µg/ml, but there was no increase in the number of micronuclei in binucleated cells in the absence of S9 (Figure 9.3B). In the presence of S9, an increase in the number of micronuclei was observed at the highest concentration tested, i.e. 50 µg/ml. Above this concentration, cytotoxicity was observed.

Figure 9.3A: *In vitro micronucleus test results, with and without S9, of investigated substances with positive prediction in silico. Results are expressed as change in number MN/2000 binucleated cells (% of NC) and cytotoxicity. *Statistically significant compared to solvent control (p<0.05).*

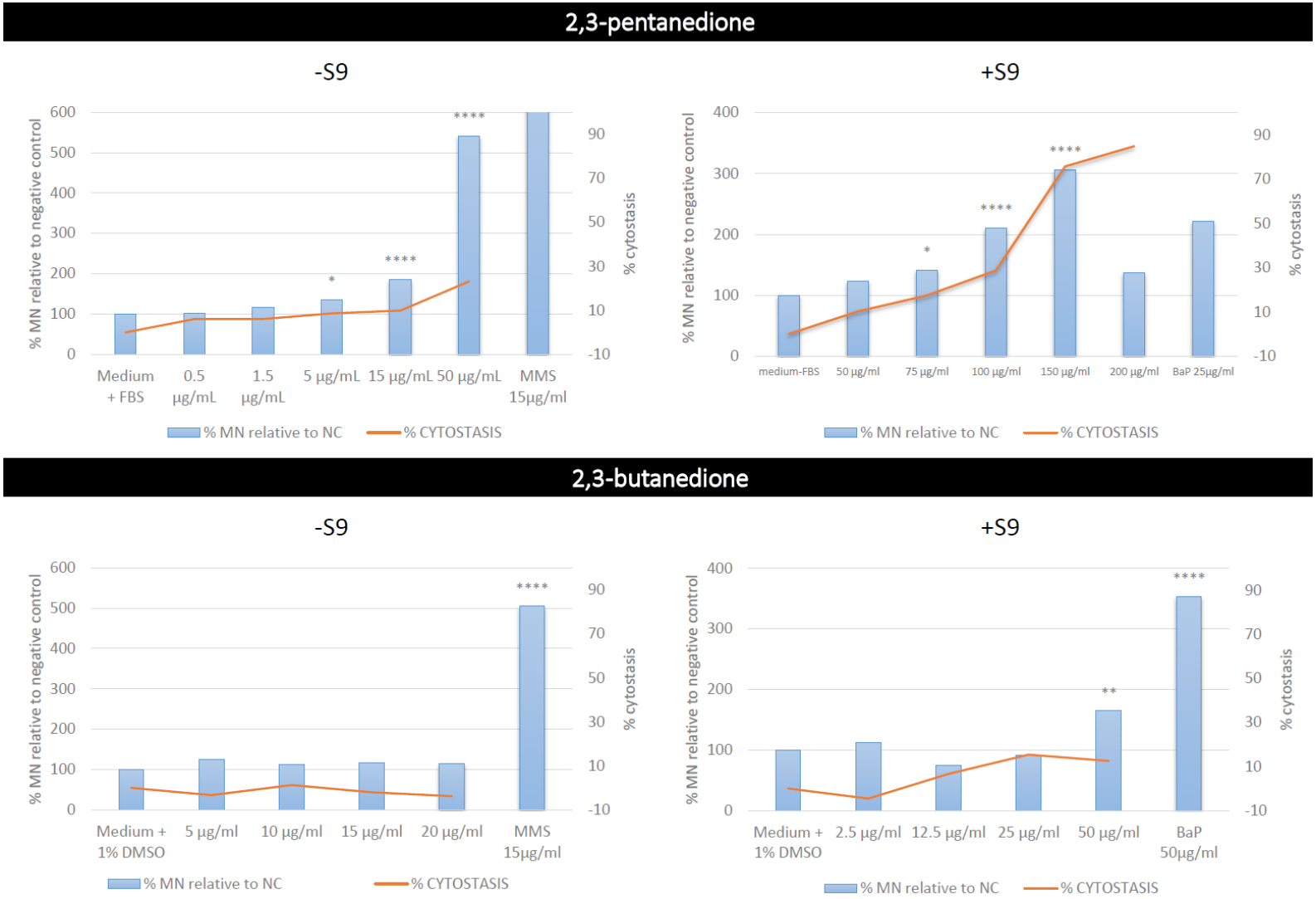
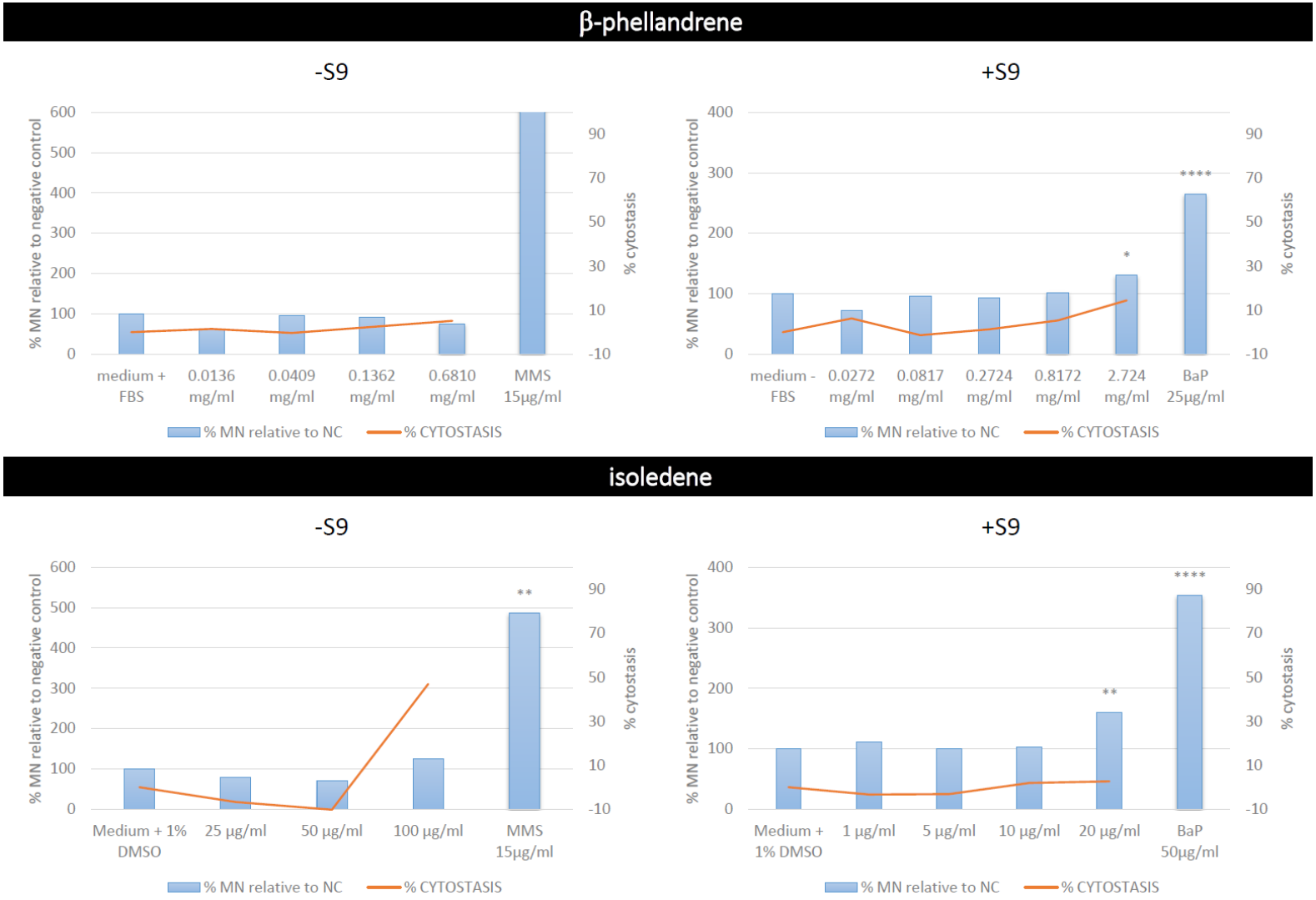


Figure 9.3B: *In vitro micronucleus test results, with and without S9, of investigated substances with positive prediction in silico. Results are expressed as change in number MN/2000 binucleated cells (% of NC) and cytotoxicity. *Statistically significant compared to solvent control (p<0.05).*



4 DISCUSSION

In this study, 129 e-liquids containing a wide variety of flavours were analytically screened by GC-MS. As expected, many different chemicals were detected in the e-liquids, including flavourings as well as 'contaminants/extractables and (tobacco) impurities'. Besides the typical synthetic flavourings, natural extracts or even essential oils are also added as flavouring to e-liquids. These may be of concern for human health as their composition is often not well characterized. Moreover, inhalation of both essential oils and natural extracts has generally been associated with a number of adverse human health effects [58]. For essential oils, sensitization is one of the major risks after inhalatory exposure [59]. Yet, other adverse health effects have been correlated with exposure to certain components present in essential oils and natural extracts such as neurotoxicity [60] (convulsions, head aches), teratogenicity and abortifacient properties [61]. Next to essential oils, some perfumes and fragrances, also used as flavourings in e-liquids, are recognized as respiratory irritants that can trigger asthma [62].

In total, more than 800 different volatile chemicals were identified in the 129 e-liquids. Due to this large number, *in silico* models were used to prioritize those components that might be of genotoxic concern, with a focus on *in vitro* bacterial mutagenicity. In general, (Q)SAR predictions for this endpoint are accurate [63], although false negatives are still possible. It was found that only 10% of the components gave a positive prediction in at least one of two (Q)SAR models. In the current strategy two complementary commercial models were applied. Other *in silico* models could be used as well as long as they comply to the OECD validation principles and that they are complementary to each other. However, this does not imply that the remaining components may not induce other adverse health effects, such as pulmonary toxicity. Although in theory such toxicological effects could also be predicted *in silico*, most of the (Q)SAR models are far less developed for these endpoints [64].

Among the substances with a positive prediction for *in vitro* bacterial mutagenicity, there were contaminants/extractables and (tobacco) impurities. As the present study focused on flavourings, these types of components were excluded for the next prioritization step, leaving 44 flavouring components. For those 44 components, literature was consulted for existing safety evaluations. Harmonized CLP classifications and EU authorities' evaluations were given higher weight than the self-classifications reported in the ECHA database by industry. Based on the information present in the existing evaluations, three flavourings could be identified as known and/or potential genotoxicants and for two flavourings the genotoxicity could not be excluded. These five flavourings were found in 10 out of the 129 analyzed e-liquid samples. According to the TPD, CMR substances are not allowed

in e-liquids and thus these e-liquids are regarded as not conforming with the TPD. In Table 9.5, more details are provided on these samples.

Table 9.5: Overview of the e-liquids containing flavourings of genotoxic concern with associated flavour description.

Name	Number of samples	Flavour e-liquid	Flavour description	Brand
safrole	1	rum cola	sweet warm spicy woody floral sassafras anise	Sedansa Rum cola
estragole	4	tobacco, and mint	Sweet-herbaceous Anise-Fennel type	Whatafog Strong Mint/Ice ice baby Sedansa David Sedansa Deluxe tobacco E-liquid Deluxe tobacco
furylmethylketon	2	tobacco, wood, hints of spices and honey	sweet musty caramel brown bread crust, balsamic Used in Chocolate, Coffee, Roast Nut, Bread, Rum, Whiskey, Tamarind, Tea and Tobacco flavours, as a trace background note.	Mistervape Maxx blend Sedansa Chocolate
transhexenal	2	apple-cinnamon	fresh, green, and natural topnote in fruity floral types.	One hit wonder - Muffin Man Twelve Monkeys - Kanzi
2,5-dimethyl-4-hydroxy-3(2H)-furanone	1	strawberry	sweet cotton candy caramel strawberry sugar	Savourea Ice strawberry

For 23 substances, the genotoxic concern could be excluded based on available data. Importantly, for 16 of the remaining 44 components with a positive *in silico* prediction for *in vitro* bacterial mutagenicity, no genotoxicity data was available in the consulted EU databases. In order to know whether the respective e-liquids are compliant with the TPD, the genotoxic profile of these components had to be evaluated further. Therefore, two *in vitro* genotoxicity tests were performed, however, this was only possible for the commercially available compounds. However, it should be noted that the Ames test was only performed in 2 bacterial tester strains. In order to conclude that a compound does not induce gene mutations in bacteria, it should be negative in all 5 strains recommended in OECD TG471.

β-Phellandrene was negative in the Ames test and slightly positive in the *in vitro* micronucleus test, whereas **isoledene**, **2,3-pentanedione and 2,3-butanedione** were positive in both tests. The last two mentioned flavourings are found in several e-liquids and are controversial as they have shown to be responsible for developing bronchiolitis obliterans in chronic inhalation studies [15]. For 3 out of these

4 components, the *in vitro* genotoxicity testing thus confirmed the genotoxic concern raised by the *in silico* predictions. For these compounds, additional tests are needed to assess whether the genotoxic effect will also occur *in vivo*. There were also indications in our *in vitro* experiments that β-phellandrene might be genotoxic. Therefore, further testing is required for this compound before a final conclusion on its mutagenic potential can be drawn. Substances with a positive prediction for bacterial mutagenicity, but for which no genotoxicity data were found and that were not commercially available, might also have genotoxic properties and thus represent a problem. This group of substances represent mainly substances from natural herbal and tobacco extracts. An alternative approach to evaluate the genotoxicity of these substances could be to test the extract as such or to test the e-liquid containing the substance instead of the pure compound. However, testing of extracts or e-liquids also poses different challenges such as their varying composition and possible matrix effects.

It is important to note that in this study, only the e-liquids were analytically screened and not the aerosols produced by heating of the e-liquid. Humans are not directly exposed to the e-liquid as such (except via unintentional oral or dermal contact), and consequently, from a human health point perspective, testing of aerosol emissions would provide more accurate information on the e-cigarette components to which humans are actually exposed to. However, the aerosol emissions may also contain other chemicals (e.g. leaching heavy metals) and decomposition products that are not present in the e-liquid itself [25], [26]. Vapourized e-liquids may therefore be more or less harmful than their liquid form. Yet, as shown in the present study in the context of priority setting and establishing a pragmatic way to check compliance with the TPD regulations, analysis of the e-liquids instead of the aerosol emissions might be more appropriate.

Some argue that by using food grade flavourings in e-cigarettes, the risks are minimized [29]. However, this is not necessarily true as the safety assessment of food flavourings is based on concentrations to which the consumer is orally exposed and these may significantly differ from the concentrations to which consumers are exposed via inhalation. The large surface area in the lungs and the absence of an epithelial barrier comparable with the gastrointestinal mucosa or to stratum corneum barrier function of the skin usually results in a higher percentage of absorption after inhalatory exposure and consequently, a higher internal dose. Also, the kinetic processes for a compound after oral exposure are different compared to those after inhalation. Hence, it is important to execute a separate risk assessment for inhalatory exposure to flavourings present in e-liquids based on their concentration in the aerosol emissions and their toxicity both at the first site of contact and after systemic uptake via the lungs [1].

Although there is a legislative framework for e-cigarettes, monitoring the compliance of e-liquids with the TPD is very difficult. One of the basic requirements is the prohibition of CMR substances in e-liquids. However, as illustrated in this study, some e-liquids do contain (potentially) genotoxic compounds. Secondly, manufacturers are legally obliged to list all ingredients, their concentrations and their toxicological data in the product notification to the authorities. This study illustrates that in practice, components are used as flavourings in e-liquids without knowledge of their genotoxic potential. Hence, these flavourings are either present in concentrations below 0.1% and thus the manufacturer is not required to include that type of toxicological data in the notification. Or, alternatively, natural extract mixtures are used for which it is difficult to obtain toxicological information by the e-liquid manufacturer.

Overall, more safety measures are needed with respect to the use of flavourings in e-liquids. Some manufacturers do have the knowledge to chemically screen and identify large amounts of ingredients or otherwise have the resources to demand the full chemical and toxicological characterization of the flavourings from the supplier. Authorities should monitor all notified e-liquid ingredients periodically, not only the registered ingredients in the notification dossier, but also unknown substances from extracts should be analytically screened and identified. Collaboration with other fields such as the food and flavor industry, the perfume and fragrance companies and herbal medicine producers should be promoted. Finally, the development of a list with flavourings that are allowed/permitted to be used in e-liquids is highly recommended.

5 CONCLUSION

To assure a minimal safety of flavourings present in e-cigarettes, the TPD states that the used ingredients may not have CMR properties. In this study, the 44 flavouring substances identified in 129 e-liquids available on the Belgian market were assessed for their genotoxic potential. Following a prioritization strategy based on *in silico* prediction tools and EU database consultation, we identified five flavouring components of high genotoxic concern (i.e. estragole, safrole, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone, furylmethylketon and transhexenal). On the other hand, a genotoxic concern for 23 of the 44 flavouring compounds flagged with a genotoxic alert by the *in silico* models could be excluded. Yet, for the 16 other flavouring substances, no *in vitro* nor *in vivo* data was available for the genotoxic endpoint. For four of the latter substances i.e. 2,3-butanedione, 2,3-pentanedione, isoledene and β-phellandrene, our *in vitro* tests indicate mutagenicity and/or the induction of chromosomal damage.

Overall, these results clearly raise concern regarding e-cigarette use and argue for more research to assess the safety of flavouring ingredients for which genotoxicity data is currently lacking. Meanwhile, from the precautionary principle perspective, these compounds should be restricted until more information becomes available. A list of restricted ingredients (chemical substances, but also natural extracts and essential oils) similar to e.g. Cosmetics regulations is necessary to assure the safety of the e-cigarette as an alternative and harm-reduction opportunity for tobacco smokers.

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Conclusions, future perspectives and recommendations

CONCLUSION AND FUTURE PERSPECTIVES

This project started at the end of 2015 when the e-cigarette was just gaining popularity in Belgium. Consequently, the Belgian Ministry of Health asked the Superior Health Council for an advice and risk assessment of e-cigarettes. This advice was required to help politicians and public health authorities with the implementation of the revised Tobacco Product Directive (2014/40/EU) that entered into force on 19 May 2014 and would become mandatory for all EU Member States on 20 May 2016. One of the recommendations, at that time, was to perform more research on the toxicity of ingredients present in e-liquids [1]. Therefore, Sciensano, previously known as the Institute of Public Health, started the collaborative research project e-SMOKE with the Vrije Universiteit Brussel (VUB) to focus on the chemical and toxicological aspects of e-cigarettes on the Belgian market.

The specific goals of this PhD project were to develop new detection and quantification methods to characterize the chemical substances present in e-liquids available on the Belgian market (Part I and II) and to assess human health hazards associated with the inhalation of flavours, present in those e-liquids, with the focus on mutagenicity/genotoxicity (Part III).

At the start of this project, information on the chemical characterization of e-cigarettes and toxicity studies related to the use of these products was very limited. Initial studies mainly focused on the comparison between (first generation) e-cigarettes and tobacco cigarettes. The targeted analytical focus on Harmful and Potentially Harmful Constituents (HPHCs) of tobacco smoke in e-cigarettes impeded the full characterization of unknown constituents specific for the e-cigarette. Over time, more analytical methods became available, each with their advantages and their drawbacks which were discussed in Chapter IV. However, there was still a need for standardized and validated methods. Indeed, this lack of standardized methods is probably one of the major factors contributing to the many discrepancies that exist between the different studies. Additionally, the difference in quality of e-liquids is also a likely contributing factor. Nevertheless, the use of methodologies comparable in sensitivity and robustness would greatly aid to generate a better and unbiased picture of the current situation. Meanwhile, bearing in mind the caveats of the utilized methodologies, an overview of the current literature about the composition of e-cigarettes could be generated. This information was condensed into Chapter III where we also formulated three problems regarding e-cigarettes that further served as a basis for a strategy to assess the quality and safety of e-liquids. These three problems are (i) discrepancies with the nicotine labelling, (ii) the presence of toxic inhalation flavourings diacetyl and acetylpropionyl, and (iii) the presence of volatile organic compounds (VOCs) and impurities. To address these issues, alternative analytical methods were developed and validated for the analysis of e-liquids.

In Chapter VI, a methodology was developed for the analysis of nicotine and related impurities in e-liquids. Although different methods have been published to measure nicotine and its impurity levels, the majority of them use a targeted LC-MS/MS approach, which is a very sensitive technique not easy to implement as routine analysis. Therefore, a more robust quantification method easy to implement in most control (industrial and governmental) laboratories is required. To meet those needs, a simple dilute-and-shoot UHPLC-DAD method for the simultaneous identification and quantification of nicotine and its alkaloid impurities in electronic cigarette refills, was developed.

Next, we also developed and validated an identification and quantification method for diacetyl and acetylpropionyl in e-liquids (Chapter V) by utilising a HS/GC-MS-based methodology. This method was fully validated using the 'total error' approach. The main advantage of this newly developed method compared to the already developed methodologies resides in the fact that the method is less labour intensive (no need for prior derivatization and no extensive clean-up) and less damaging for the column and the instrument due to the use of the headspace technique. However, the developed quantitative GC-method still requires the use of matrix-matched calibration, because of the presence of the e-liquid matrix which is oily and highly viscous, leading to differences between assay determination of duplicates. Further research should focus on the sample preparation of e-liquids to eliminate the matrix effect and as such will allow even more robust and precise measurements. During this project, we also started the development of a liquid-liquid micro extraction for e-liquid matrix based on freezing drop. Also other methodologies should be explored such as solid-phase (micro)extraction (SP(M)E). Thus, for future analytical methods it will be important to focus on an appropriate sample preparation.

Next to these two quantification methods, we have also developed methods that are applicable for the screening of flavourings, VOCs and the additives caffeine and taurine in e-liquids. It should be noted that in the case of untargeted flavouring screening with the GC-MS, an accurate identification of all components was not possible. Unidentified peaks should be further investigated using high-resolution MS-techniques for the elucidation of the structure, as these might represent potential interaction by-products.

The developed analytical procedures from Chapter V and VI, which are the basis for a strategy to assess the quality, were applied to analyse 246 e-liquid samples available on the Belgian market (Chapter VII). More precisely, we investigated whether the regulatory changes introduced in 2016 had an impact on the quality of the refill e-liquids through analysis of their chemical composition. Hence, the nicotine concentration accuracy was investigated in samples before, during and after the implementation of the revised TPD as an indicator of good manufacturing practices. Also the nicotine-

related impurities, VOCs, the additives caffeine and taurine and the flavours diacetyl and acetylpropionyl were quantified. Taken together, our results demonstrate that on the Belgian market the overall quality of e-liquids is nowadays better than before the implementation of the TPD. Vapeshop samples are generally more compliant to the TPD than internet samples and DIY samples. The market study also confirmed that continuous monitoring of the e-liquid market remains important, together with targeted measurements to assure good e-liquid products (in the context of harm reduction) such as the use of high quality starting materials and good manufacturing practices and stability testing for appropriate storage packaging. The market study also showed that diacetyl and acetylpropionyl are still significantly present in e-liquids, despite concerns about their inhalation toxicity. For this matter, the legislation is too vague and diacetyl is not (yet) banned as such in e-liquids.

We also acknowledge that other substances besides nicotine, nicotine-related impurities, VOCs, caffeine and taurine should be investigated as well. This includes the screening of heavy metals, although this is considered only relevant in the case of cartridges. Yet, other additives such as vitamin E acetate should be included since it is associated with e-cigarette or vaping product use-associated lung injury (EVALI). Additionally, also more focus is required on the detection of illicit additives e.g. tetrahydrocannabinol (THC) in e-liquids containing cannabidiol (CBD), which are gaining an enormous popularity in Belgium, possibly because of the regulatory gaps in the TPD.

In the end, it would be useful to create a kind of registration file with the specifications to which e-liquids and e-cigarettes need to comply, similar to a pharmacopoeia monograph. These monographs should be accompanied by standardized methods by which they are analysed. In anticipation of these guidelines from the health authorities or regulating agencies, the industry has already started to develop such methods. The AEMSA (American E-Liquid Manufacturing Standards Association) and ANFOR (Association Française de Standardisation) have put forward some guidelines to assess the e-liquids in order to be compliant with the TPD. However, it stands to reason that these guidelines should be reviewed by an independent organization, preferably governmental to assure the reliability. Indeed, recently the ISO/CEN taskforce for the standardization for e-cigarettes and e-liquids has been set up. In this working group governmental laboratories, the industry and universities are represented to collaborate towards the development of such standards. In parallel with this working group, a collaborative network of European laboratories has joined a task force in order to develop validated methods as well that can be used by the authorities to monitor the e-liquid market.

From our market study we know that flavourings like diacetyl and acetylpropionyl are still significantly present in e-liquids. However, little to no knowledge is available on whether they actually pose a risk in the concentrations that they are present in e-cigarettes. Therefore, as a case study, a risk

assessment was carried out for the consumption of e-liquids containing the flavouring diacetyl (Chapter VIII). The proposed methodology to assess the risk for diacetyl via e-cigarette use was based on a previously published assessment of RIVM. Our outcome showed that there is no risk for systemic effects. However, the risk could not be excluded for local lung toxicity after repeated exposure. Hence, we recommend to avoid the use of diacetyl as flavouring in e-liquids.

The utilised risk assessment method for e-cigarette use can also be applied for other e-liquids ingredients than flavourings. The model can, however, be substantially improved on different levels. For example, more information is needed on the use of e-cigarettes in different population groups (smokers, non-smokers, ex-smokers, heavy vapers, etc.). This also includes more up-to-date information about the topography of e-cigarette users to predict the exposed dose of the assessed substances. Furthermore, more accurate modelling of the inhalation of e-cigarettes will allow better predictions, especially when more PBPK modelling is involved. In a next stage, these risk assessments cases serve for the calculations of concentration thresholds and limits of certain substances in e-liquids.

Further in this thesis, we focused more on the toxicological aspect of flavourings present in e-liquids and more specifically their mutagenic/genotoxic properties (Chapter IX). Because of the large number of available flavourings in e-liquids, a screening strategy has been proposed to prioritize the flavourings of highest concern for human health. This prioritization strategy for identifying potentially genotoxic substances in e-liquid flavours, is based on a combination of analytical screening, *in silico* prediction and literature consultation. We identified five substances in e-liquids with proven genotoxic properties including estragole, safrole, 2-furylmethylketon, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone and transhexanal. For 16 other substances however, no *in vitro* nor *in vivo* data was available, thus no conclusive evaluation of genotoxicity was possible for these substances. Most of these components are components that can be found in natural extracts such as essential oils and tobacco extracts. From those 16 substances four compounds were commercially available (2,3-butanedione, 2,3-pentanedione, isoledene and β-phellandrene) and could be tested *in vitro* for their mutagenicity and potential chromosomal aberration damage. As an alternative approach for those substances for which no commercial standard is available, the whole e-liquid could be tested to evaluate the genotoxicity, provided that known genotoxic compounds are absent. Nevertheless, from our data, it can be stated that more investigation is needed to assure the safety of ingredients for which today no genotoxicity data are available. The findings clearly indicate that the use of flavouring components might pose a potential health risk for e-cigarette users. Moreover, the proposed working strategy demonstrates the

importance of the use of animal-free alternative methods for further safety assessment of e-cigarettes [2].

Genotoxicity is an important toxicological endpoint that is present in the legislation of all consumer products coming on the EU market. There are, however, other crucial toxicological endpoints for smoking products such as local lung toxicity and systemic toxicity. Hence, it could well be that, similar to tobacco smoke, e-cigarette aerosol is also associated with respiratory inflammatory diseases. Therefore, the correlation between long-term e-cigarette use and lung inflammatory diseases should be investigated in more detail. In this context, alternative *in vitro* models including lung cells cultured at the air-liquid interface (ALI) could be of great help to investigate potential lung toxicity and cell-cell interactions and mechanisms involved [3].

So far the presented results only reflect the e-liquids itself since the aerosols of e-cigarettes were not investigated. In the future, more focus should be put on the development of appropriate methods for the investigation of emission aerosols. Especially, attention should be given to the development of efficient trapping methods and the further optimization of topography models that mimic as much as possible real-life conditions. Therefore, considerably more work needs to be done for the harmonization of emission studies of e-cigarette aerosols. Moreover, several other aspects still need to be explored: the process of heating, the interactions between the different components, the formation of other (hazardous) components, inhalatory toxicity studies and the effect of mixture toxicology.

IMPACT AND RECOMMENDATIONS

The results obtained in this PhD thesis may contribute to the highly needed scientific data to draft and/or adapt the legislation with respect to *the quality and safety of e-liquids*. The following recommendations could be formulated to the public health sector and regulatory agencies.

The current legislation has already covered a great part to assure the quality and safety of e-cigarettes. The sale of e-cigarettes is well regulated in Belgium where internet sale is forbidden. Results of the market study confirm the necessity of this measure. Indeed, a comparison between the products coming from the regular, illegal and internet market was conducted. The obtained results support both the national (DG4, DG5, FAMHP) as well as the European authorities (EDQM) in their campaigns to raise public awareness for the dangers of e-cigarettes and more general all products bought on illegal markets or from internet sites that are not linked to known e-cigarette brands or registered vapeshops.

However, besides confirming the actions taken under the current legislation, some **measures still need some fine-tuning**. One of the gaps in the e-cigarette legislation is that it only applies for nicotine-containing e-cigarettes. Yet, the presence of VOCs of toxic flavourings in e-liquids that are forbidden in nicotine-containing e-liquids remain a health concern for non-nicotine containing e-liquids. Therefore, it would be wise to extend this prohibition also for non-nicotine containing e-liquids.

This legislative loophole of unregulated non-nicotine containing e-liquids can be used as a way to circumvent the e-liquid requirements in the TPD. There is anecdotal evidence from tabacologues that non-nicotine e-liquids are used with so called “nicotine-boosters” which are highly concentrated nicotine (20mg/ml) e-liquids without flavourings. The combination of both are the DIY e-liquids, which turned out to have serious non-conformities when assessed with our quality strategy. Thus, the TPD should further be extended to non-nicotine containing e-liquids in order to prevent these bad practices.

Also in the current e-cigarette legislation, ingredients are allowed to be used in e-cigarettes as long as there is no clear evidence of toxicity. We therefore suggest that from a regulatory point of view, the use of flavourings and additives, for which limited to no toxicological data is available, should be restricted because of the unknown health risk they could pose. Registration of flavourings in positive and negative lists (as done for cosmetics) would also be a helpful tool to avoid exposure of the consumer to substances with unknown inhalation toxicity, awaiting their full toxicity assessment. A list of restricted ingredients (chemical substances, natural extracts and essential oils) is also pivotal to assure the maximum safety for e-cigarette use. In this way also the number of flavourings would be

restricted which would in turn reduce the attractiveness of e-cigarettes. Lastly, this list would also help to monitor the e-liquid market and make inspections more easy.

In addition, it is necessary to address some **measures that are currently missing** in the legislation. There are still no requirements concerning the stability of e-liquid products and the use of expiry dates. The results obtained from the preliminary stability study we have conducted showed that the stability of nicotine cannot be guaranteed after six months, as the nicotine related impurities were significantly increasing. A more extensive stability study for each product would be necessary (and should be required) to assure the quality of the product, as the e-liquid composition might influence the stability. Based on these studies, an appropriate packaging should be chosen (protection from light and humidity) and well-supported expiry dates should be provided to assure the quality and hence safety of the e-liquid products.

Lastly, it is equally important to anticipate the introduction of alternative, new electronic nicotine delivery systems in the e-cigarette legislation before they become available on the EU and Belgian market. Indeed, the e-cigarette continuously evolves, both with respect to the devices used to produce the aerosols and the ingredients in the e-liquids. This does, however, not necessarily mean that these "new" products are safer. One of the latest examples in this context are "pod mods" such as JUUL.

Besides the policy makers and health authorities, the **obtained information can also help officials and health practitioners** responsible for communication and education within the framework of tobacco control policy to formulate scientifically based recommendations concerning e-cigarettes. Indeed, the obtained results indicate that e-cigarettes are still associated with certain health concerns that are not yet fully cleared. Therefore, from a health risk-benefit point-of-view, it would be wise to only use e-cigarettes as an alternative smoking cessation tool if other therapies wouldn't be successful to quit smoking.

Finally, the **strategies used in this project** (analytical assessment, genotoxicity assessment using non-animal methods and risk assessment) **could be adopted by the industry** to support their development of adapted, new and "safer" products, with less concerns for public health.

EPILOGUE

During the e-SMOKE project the most frequently asked question that was posed was: “are e-cigarettes safe?”. This is a very difficult question to answer since e-cigarettes have been a huge point of contention within the scientific community. On the one hand some scientists are convinced that the e-cigarette is the answer to eliminate the more deadly addiction of tobacco smoking, while on the other side some scientists believe that the e-cigarette will only facilitate the habit of tobacco smoking and will lead to the renormalization of nicotine-addiction.

With this research project we tried to contribute to a small piece of the answer to that specific question. We showed that over the years, the e-liquid quality is changing in the positive sense, with less nicotine mislabeling and less impurities and contaminants. However, one of the potential unknowns introduced with the e-cigarette, is the use of different flavours. The direct and intentional deep inhalation of these substances is not without risk as demonstrated in this project through the risk assessment of diacetyl and the genotoxicity testing of flavours.

Yet, the assessment of potential inhalation toxicity of e-cigarette ingredients is only one piece of the answer. Studies on the long-term effects of e-cigarette use, the clinical evidence for e-cigarettes as a smoking cessation tool, the effect of addictiveness of flavours and many more are out of scope for this thesis, but are equally necessary. In general, it can be concluded that more research is still needed not only to improve the quality and content of the e-cigarette as known today, but also to maintain high standards for the new generations of e-cigarettes that are continuously evolving.

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I have this cartoon on my office wall that says “my thesis is written in blood, sweat, tears and coffee”. It indeed took some sweat during the hot summers, it certainly took tears and I don’t exaggerate if I say it took at least 540 L of coffee (estimation based on three cups a day). The only reason I survived was because I was surrounded with amazing people

Doing a PhD with three promotors might sound like a source of frustration. However, I was lucky that my promotors were often on the same page and complemented each other. **Eric**, thank you for giving me the opportunity to start this project at Sciensano and for keeping me there. I would also like to thank you for your support and patience, especially the past year. **Tamara**, thank you for having patience with my writing and help me grow to an independent researcher. **Vera**, your enthusiasm and passion for science was contagious and motivating.

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Sophia

CURRICULUM VITAE

Sophia Barhdadi was born on the 2nd of July 1990 in Borgerhout (Belgium). She attended secondary school direction Latin-Mathematics at *Sint-Lievenscollege* in Antwerp, Belgium. In 2013, she obtained a Master's degree in pharmaceutical sciences (major drug development) with distinction from the Ghent University (UGhent) (Ghent, Belgium) which included a 4-month international exchange at Chemiphar laboratories in Kampala, Uganda for the preparation of her Master's thesis entitled 'Detection of falsified anti-malarial drugs with HPTLC-densitometry' under the promotorship of Em. Prof. dr. Willy Lambert.

Hereafter, Sophia started the interuniversity advanced Master program in industrial pharmacy of UGhent, KULeuven, UA and VUB. Her master thesis entiteled 'Development of a hGHAb-based Surface Acoustic Wave method for the functional quality characterization of NOTA-modified somatropins' was conducted at the Laboratory of Drug Quality and Registration (DruQuaR) in Ghent University under the promotorship of Prof. dr. Bart De Spiegeleer. She graduated in 2014 with high distinction. To obtain her license for Qualified Person, she finished a 6-month internship between September 2014 and February 2015 in the QC laboratories at Janssen Pharmaceutica in Beerse (Belgium). Thereafter, she was employed as a QA associate in the laboratories of Pharmaceutical Development and Manufacturing Sciences departement of Janssen Pharmaceutica in Beerse (Belgium) from March 2015 until October 2016.

Sophia started her PhD project about the chemical and toxicologicial assessment of e-cigarette liquids in November 2016, which was a collaboration between the Medicines and Health Products section of the former Scientific Institute of Public Health, now merged into Sciensano and the department of In Vitro Toxicology and Dermato-Cosmetology (IVTD) of the VUB under the joint promotorship of Dr. Eric Deconinck, Prof. Tamara Vanhaecke and Prof. Vera Rogiers.

Sophia presented her results at several international and national conferences. She authored 5 scientific publications in international peer-reviewed journals of which 3 as first author. Furthermore she supervised 3 Master's thesis projects. Sophia also stayed several months at the National Institute for Public Health and the Environment (RIVM) in Bilthoven, the Netherlands to work with smoking machines for future reseach of e-cigarette products. Currently, Sophia is working as a scientist at Sciensano and is one of the members of experts of the Superior Health Council regarding e-cigarettes.

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