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# Scientific-technical support activities to DG TAXUD-C-2 on the option to include e-cigarettes within the scope of excisable goods for the Impact Assessment on a possible revision of Directive 2011/64/EU

*Administrative Arrangement  
TAXUD/2015/DE/329*

*Final report*

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## Table of contents

Acknowledgements.....	3
Executive summary .....	4
Introduction .....	5
Determination of nicotine and nicotine related alkaloids in commercial e-liquids.....	7
1.1    Samples .....	7
1.1.1    Sample preparation and analysis .....	7
1.1.2    Tobacco alkaloid content of liquids used in e-cigarettes .....	9
Volatile profiles of tobacco and non-tobacco flavoured e-liquids .....	13
1.2    Samples .....	14
1.2.1    Sample preparation and analysis .....	14
1.3    Data Analysis .....	14
1.3.1    Classification of samples.....	15
1.3.1.1    Partial least square discrimination.....	16
1.3.1.2    Decision tree.....	18
Conclusion.....	20
ANNEX 1 – Analytical method for the determination of tobacco alkaloids in e-liquids, test samples, and results.....	21
A1.1.    Analysis of tobacco alkaloids by HPLC-MS/MS in HILIC mode.....	21
A1.2.    Test samples .....	26
A1.3.    Contents of tobacco alkaloids determined in e-liquid test samples .....	34
ANNEX 2 – Analytical method for the evaluation of flavour profiles .....	44
A2.1.    Analysis of volatile constituents of e-liquids by thermal desorption high-resolution gas chromatography QTOF mass spectrometry.....	44
A2.2.    Test samples .....	46
A2.3.    Compound identification .....	49
References .....	53
List of figures.....	55
List of tables.....	56

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## Executive summary

Electronic-cigarettes are electrical devices that produce an inhalable aerosol from a special liquid. The liquids (e-liquids) may consist of a solution of nicotine and flavourings in an appropriate solvent. Nicotine-free products are available as well. A large range of differently flavoured e-liquids is offered to the consumer. The market of electronic cigarettes and corresponding e-liquids experienced in recent years enormous growth. Some EU Member States reacted on this development and introduced excise duties on e-liquids.

The partial introduction of excise duties in the common market and the lack of harmonisation of their magnitude might cause distortions of the market. For that reason, the Directorate-General Taxation and Customs Union (DG TAXUD) commissioned a study on the identification and characterisation of e-liquids, which should be based on substances naturally occurring in tobacco.

The Administrative Arrangement (AA) with DG TAXUD foresaw the determination of the concentrations of nicotine and related tobacco alkaloids in e-liquid samples, and to evaluate the agreement of the measured concentrations with concentrations indicated on the product label.

Additionally, the possibility of identifying e-liquids containing tobacco derived flavours had to be studied with the aim to use this information for product classification.

More than 200 e-liquid samples acquired in different countries were analysed for their tobacco alkaloid contents by liquid chromatography tandem mass spectrometry. A high proportion of the investigated products contained nicotine at a level significantly different from the indicated concentration. Mostly lower concentrations were found. However, significant amounts of nicotine were determined in some products declared as "zero-nicotine".

Volatile profiles of 108 e-liquid samples were measured by gas chromatography hyphenated to high-resolution quadrupole time-of-flight (QTOF) mass spectrometry. The data obtained from the analysis of was investigated with multivariate statistical methods for possibilities of discriminating tobacco- and non-tobacco flavoured e-liquids. The developed statistical models allowed identification of tobacco derived products with high probability.

If a harmonized taxation of e-liquids were to be proposed, the following considerations should be taken into account in designing the tax base:

- The declared nicotine content often does not match with the measured nicotine content;
- Moreover, in products declared as "zero-nicotine" significant amounts of nicotine can be found;
- Chemical analysis allows distinguishing tobacco and non-tobacco e-liquids with high probability.

# Introduction

## General information about e-cigarettes

Electronic (e-) cigarettes are battery-powered hand-held devices that convert a liquid into an inhalable aerosol. Attempts to produce and commercialise such aerosol dispensers are known for a long time (Robinson, 1930).

However, the currently marketed e-cigarettes are based on an invention made in 2004 by the Chinese pharmacist Lik Hon (Hon, 2004). He designed a battery-powered device that aimed to supply nicotine to smokers without exposing them to the tar component of conventional smoking. E-cigarettes typically consist of a mouthpiece, an atomizer, a battery, and a tank that contains the liquid for vaporization ("e-liquid") (Famele et al., 2015). The atomizer is a heating element forming a fine mist from the e-liquid which carries the flavour, and which provides a sensation of smoking to the user. E-cigarettes might be manufactured for single use only, or might contain a replaceable or refillable e-liquid tank, and a rechargeable battery.

The lack of standardization has led to various designs of e-cigarettes. Some of the e-cigarettes are in size close to tobacco containing cigarettes, while others are much larger and heavier. Similarly, the number and composition of refill solutions grew in the last decade exponentially. In contrast to tobacco containing cigarettes, for which EU legislation prohibits characterising flavours, e-liquids are marketed with a broad range of flavours and each of them is usually available with different levels of nicotine content (EU, 2014). It should be stressed that only a part of the commercialized e-liquids smells of tobacco. A big proportion of the market comprises e-liquids with non-tobacco flavours. These might taste like alcoholic and non-alcoholic beverages (coffee, wine, black tea, brandy, lemonade...), sweets (candy, cookies, chocolate, muffins...), or all kinds of fruits (cherry, strawberry, apple...).

The non-tobacco flavoured e-liquids are usually manufactured from flavourings that are GRAS (generally recognized as safe) certified by the Flavour & Extract Manufacturers Association (FEMA). However, it has to be noted that this certificate relates only to the use of the flavour as a food additive, and to exposure via food.

Tobacco flavours are mainly produced via the extraction of tobacco. Residues of the manufacturing of cigarettes and other tobacco products, such as leaf stems, may be used for that purpose. Technologies applied for the extraction of tobacco flavour comprise amongst others liquid extraction, supercritical fluid extraction, and steam distillation.

Nicotine in e-liquids is extracted from tobacco and this process may also extract other minor tobacco alkaloids, such as nornicotine, anatabine, anabasine and myosmine and cause the formation of degradation products of nicotine such  $\beta$ -nicotyrine, cotinine and nicotine-N-oxide (Etter et al., 2013). Oxidative degradation of nicotine can also occur during the manufacturing processes of e-liquids, and therefore, high amounts of nicotine-related tobacco alkaloids can indicate the inadequate handling and storage of the product (Famele et al., 2015). In some cases, tobacco absolute extracts (also called naturally extracted tobacco (NET)) are used as additives in tobacco-flavoured e-liquids to get a more "authentic" tobacco flavour. NETs comprise tobacco extracts that are obtained by maceration in usually propylene glycole. This production process can result in higher amounts of nicotine-related impurities (Farsalinos et al., 2015). However, different research groups observed significant differences between the nicotine contents measured and declared on the product label (Cheng, 2014; Davis et al., 2015). This can be reasoned by a lack of regulations on the composition of e-liquids. The deviations might

also find their origin in the large number of small and medium scale producers, who not always have the necessary tools and procedures for a proper quality control of the production process.

### **Use of e-cigarettes**

E-cigarettes continued arising curiosity among European citizens. According to the Special Eurobarometer 429, 12% of the EU 28 population above the age of 15 years use or have used e-cigarettes, which is more than was recorded in a survey conducted in 2012 (European Commission, 2015). Four percent of smokers and three percent of ex-smokers used at the time of the survey (end 2014) e-cigarettes. Nicotine containing e-cigarettes are preferred by users

However, Eurobarometer 429 does not allow drawing conclusions on the types of flavours preferred by consumers (Giovenco et al., 2015). They reported that unflavoured and menthol flavoured e-cigarettes dominated the US e-cigarette market in 2012 and 2013. E-cigarettes sales via traditional retail channels reached within this period the level of about \$636 million. Unclear is the volume of online-traded electronic cigarettes and refill liquids.

### **Legal and fiscal background**

Electronic cigarettes and refill containers are regulated in the EU by Directive 2014/40/EU of the European Parliament and of the Council (tobacco products directive "TPD").

The TPD mandates the European Commission to detail the provisions for the notification of electronic cigarettes and refill containers as well as of technical standards for the refill mechanism of these products. The power for authorisation for placing on the market of e-liquids remains with the Member States.

E-cigarettes are currently not excisable tobacco products, as defined in Directive 2011/64/EU.

However, some EU Member States introduced or are in the process of introducing excise duties on e-liquid refills. Excise duties range from €0.01 per ml e-liquid to €0.60 per ml e-liquid. The different approaches taken in taxation of e-cigarettes and different levels of excise duties might lead to a fragmentation of the market, and might have negative impact on the functioning of the internal market. This was also expressed in a study on "measuring and reducing of administrative costs for economic operators and tax authorities and obtaining in parallel a higher level of compliance and security in imposing excise duties on tobacco products" commissioned by DG TAXUD (Stener et al., 2014). Moreover, in view of the increasing market share of e-cigarettes, replacing partially traditional tobacco products, a loss of revenue of Member States can be expected.

On 16 March 2016, the Council agreed that the market of e-cigarettes should continue to be monitored and when appropriate, the efforts to develop an efficient taxation method for such products should be intensified.

## **Terms of reference**

In the light of the possible need to develop an efficient taxation method and the potential inclusion of e-liquids in the scope of excisable products, the JRC was asked by DG TAXUD to identify and characterise components of liquids used in e-cigarettes to provide the scientific information needed for the potential harmonised implementation of fiscal measures in the Member States related to e-cigarettes. The definition of the 'characterising element' of e-cigarettes should be based on substances naturally occurring in tobacco, irrespective whether they have been extracted from tobacco leaves, synthesised or obtained from other natural sources. Data on the content of nicotine and related alkaloids of at least 150 samples had to be provided. Additionally, flavour profiles of tobacco flavoured and non-tobacco flavoured e-liquids had to be studied. Details on the implementation of the scope were discussed and agreed upon during the kick-off meeting.

## **Determination of nicotine and nicotine related alkaloids in commercial e-liquids**

### **1.1 Samples**

DG TAXUD supplied the JRC with 22 e-liquid samples. The rest of the in total 216 samples were acquired from local markets and internet shops between June 2015 and February 2016. The test samples originated from 12 EU Member States (in alphabetical order: Belgium, Croatia, Czech Republic, Finland, France, Germany, Hungary, Italy, Lithuania, Netherlands, Poland, Romania, and United Kingdom). According to labels, the e-liquids were manufactured in EU Member States (Croatia, Czech Republic, France, Germany, Hungary, Italy, Netherlands, and Spain), as well as in third-countries such as China and USA. Nicotine concentration ranged from 0 to 48 mg/mL as declared by manufacturers. For comparison purposes, several traditional cigarettes from major brands were acquired in EU local markets, as well as 3R4F research cigarettes from the University of Kentucky (Kentucky Tobacco Research & Development Center, USA).

It has to be stressed that, due to the lack of reliable data, sampling did neither take into account consumption habits and preferences of e-cigarette consumers nor market share of certain brands/products.

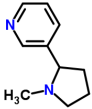
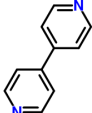
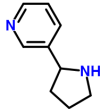

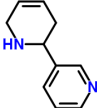
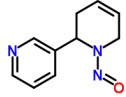
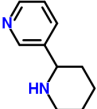
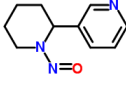
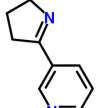
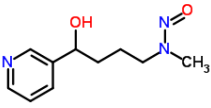
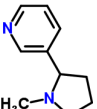
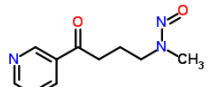
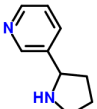

#### **1.1.1 Sample preparation and analysis**

E-liquid samples were analysed following an in-house developed method. Briefly, samples were diluted in a mixture of acetonitrile/water (50:50, v/v), filtered and analysed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS). Quantification of nicotine (NIC) and related compounds was carried out by internal standardisation with stable isotope labelled analogues. Tobacco alkaloids from cigarettes were extracted by ultrasound assisted extraction with a mixture of methanol/water (80:20, v/v). Extracts were then centrifuged, filtered and analysed following the same procedure as the e-liquid samples. Table 1 shows the



chemical structures and acronyms of the analysed compounds. Details of the analytical method are given in Annex A1.1.

**Table 1: Chemical structures and acronyms of the analysed substances**

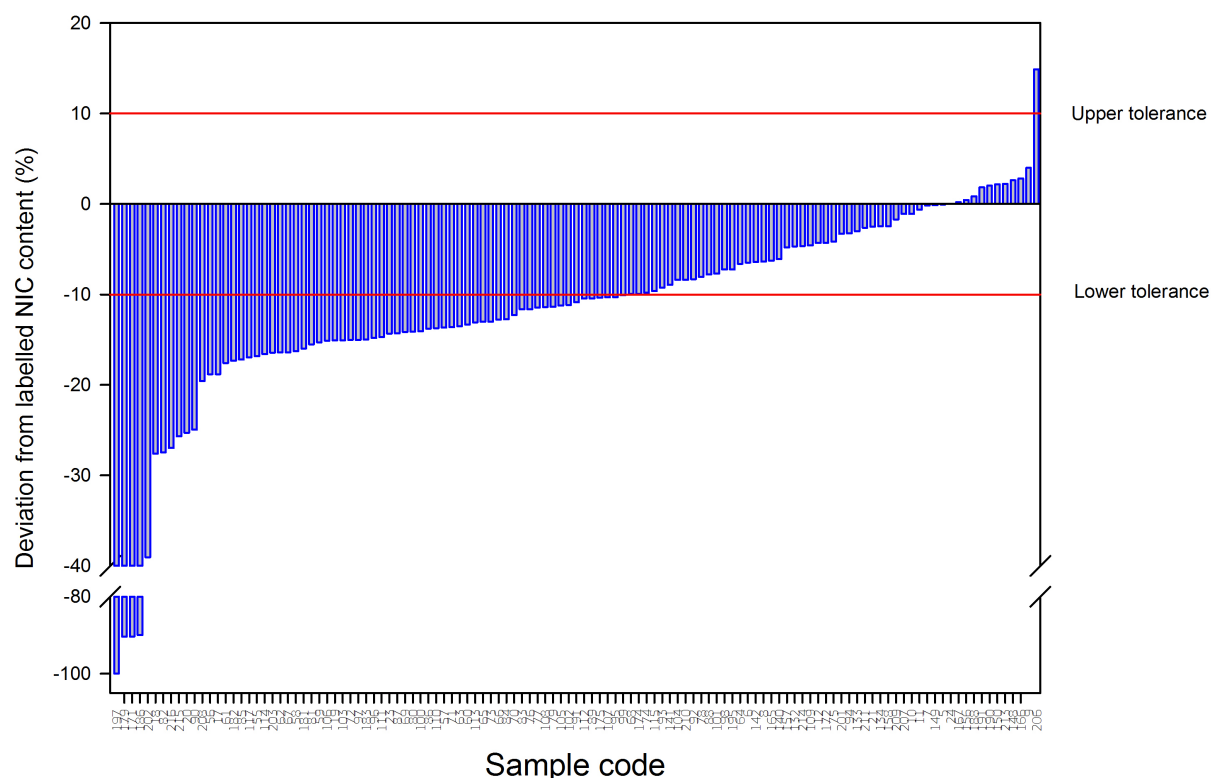
Compound	Acronym	Structure	Compound	Acronym	Structure
Nicotine	NIC		4,4'-Dipyridyl	DIPY	
Nornicotine	NNIC		N-Nitrosornicotine	NNN	
Anatabine	ATB		N-Nitrosoanatabine	NATB	
Anabasine	ABS		N-Nitrosoanabasine	NABS	
Myosmine	MYO		4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol	NNAL	
Cotinine	COT		4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	NNK	
Norcotinine	NCOT		Cytisine	CYS	

### 1.1.2 Tobacco alkaloid content of liquids used in e-cigarettes

The measured concentrations of NIC and related compounds in the analysed e-liquids are listed in Annex 1 in Table A-2. In agreement with previous literature reports (Farsalinos et al., 2015; Goniewicz et al., 2012; Peace et al., 2016), measured NIC concentrations were often significantly lower than the concentrations declared by manufacturers on the e-liquid labels (Figure 1). A slight variability of contents might be unavoidable in the manufacture of e-liquids. Therefore, guidance on tolerable deviations of the actual NIC contents from the contents declared on the product labels was taken from the British guide for manufacture, importation, testing and labelling of vaping products, including electronic cigarettes, e-liquids, e-shisha and directly-related products (BSI, 2015). This guide requires that the actual NIC contents of e-liquid preparations is within  $\pm 10\%$  of the content indicated on the label. More than half of the tested samples with declared NIC contents did not comply with the 10% threshold, suggesting a need for improvement of the production process. One sample exceeded the indicated NIC content by more than 10%, while the majority of the samples contained less than 90% of the NIC content indicated on the packaging.

Four products contained NIC at levels higher than 20 mg/ml, which is the upper limit specified in Directive 2014/40/EU (EU, 2014). However, it has to be noted that these products were produced and acquired before the TPD came into force.

**Figure 1: Difference between the NIC concentrations declared on the label and the measured concentration in the analysed samples.**



The study highlighted the presence of measurable amounts of NIC in 45 out of 91 samples labelled as "zero nicotine". For these e-liquids, NIC concentrations ranged from 0.0005 mg/ml to 13.57 mg/ml. According to the British guide for vaping products, zero-nicotine-content labelled e-liquids shall not contain more than 0.1 mg NIC per millilitre of finished product. Eight of the 91 tested zero-nicotine e-liquids exceeded the threshold of 0.1 mg NIC per millilitre of finished product.

These findings indicate that the information about NIC levels on product packages may be inaccurate, which highlights the importance of both proper manufacturing and quality control, and surveillance in order to guarantee the quality of the products on the market.

Nicotine for the preparation of e-liquids is usually derived from tobacco. As such, it usually contains also other tobacco alkaloids. While the TPD only requires using ingredients of high purity for the manufacture of nicotine-containing liquids, the British guide PAS 54115:2015 is more specific by setting the purity requirement to either European Pharmacopoeia (Ph. Eur.) or United States Pharmacopoeia (USP) grade. Both Pharmacopoeias specify the purity of NIC to at least 99%. The level of total impurities tolerated by the USP is maximum 1.00%, while Ph. Eur. set it to maximum 0.80%. Individual minor tobacco alkaloids (impurities) may not exceed 0.50% (USP) and 0.30% (Ph. Eur.) respectively.

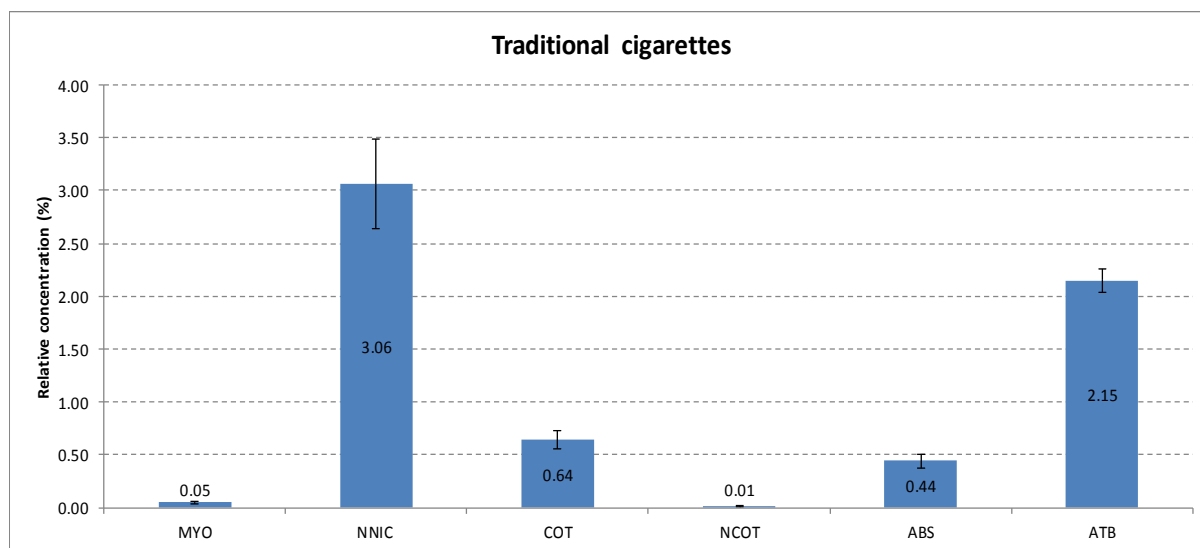
Minor tobacco alkaloids, myosmine and nornicotine were found in all tested e-liquids that contained NIC, at concentrations in the ranges 0.112-62.85 µg/mL and 0.179-26.65 µg/mL, respectively (Annex 1, Table A-2). Cotinine, anatabine and anabasine were present in most of the samples at concentration levels up to 78.62 µg/mL, 49.01 µg/mL and 40.34 µg/mL, respectively. Norcotinine was found in only 10 samples, in the range 0.010-3.010 µg/mL. The average relative contribution of individual minor tobacco alkaloids to the measured total tobacco alkaloid content (including NIC) was below 0.1 %. None of the tested samples contained N-nitroso derivatives of tobacco alkaloids.

### **Comparative evaluation of tobacco alkaloids in e-liquids and traditional tobacco**

The analysis of traditional cigarettes from several brands showed NIC as the most abundant alkaloid with concentrations ranging from 12.21 mg/g to 15.88 mg/g (Annex 1, Table A-3). All samples presented a similar pattern of minor tobacco alkaloids, with nornicotine (391.15-584.54 µg/g) and anatabine (304.75-386.60 µg/g) as the most abundant ones. Lower concentrations were obtained for cotinine, anabasine and myosmine. Norcotinine was also the least abundant alkaloid in traditional cigarette tobacco.

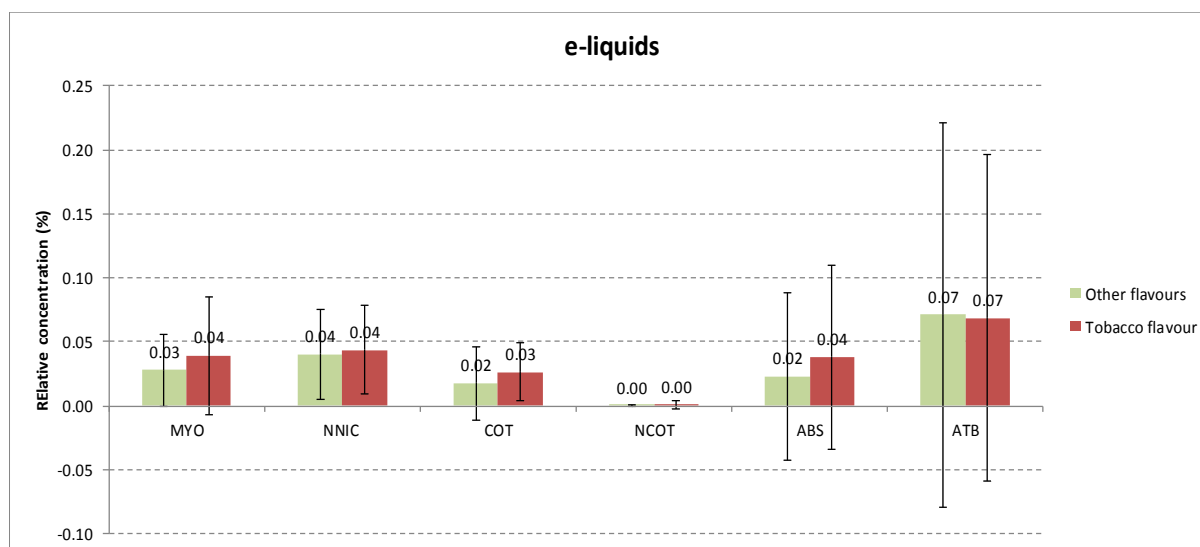
Although differences exist among the different tobacco varieties/species, there are generally certain correlations between the contents of NIC and the minor alkaloids (Sisson & Severson, 1990). As displayed in Figure 2, the profile of minor alkaloids extracted from cigarette tobacco was rather stable, despite different brands of cigarettes were analysed. Therefore, in an attempt to identify the source of NIC applied in the production of e-liquids, the relative concentrations of minor tobacco alkaloids were investigated (sum of tobacco alkaloids=100%).

**Figure 2: Relative concentrations of minor alkaloids in traditional cigarettes.**



The concentrations of these minor tobacco alkaloids are much lower in e-liquids than in traditional cigarette tobacco. However, their profiles were not as consistent as in cigarette tobacco extracts, but varied enormously. Direct comparison of the profiles obtained for tobacco-flavoured e-liquids and other nicotine-containing flavours did not show any significant difference (Figure 3). Lisko et al. (2015), who concluded that concentrations of minor alkaloids highly depend on the applied manufacturing and purification of NIC extracts, thus affecting their relative concentration in the final e-liquids, obtained similar results. Poor manufacturing practices might be another explanation for the poor correlation between NIC and minor alkaloids in e-liquids. In addition, oxidation processes may also influence the profiles of minor alkaloids. When NIC is exposed to air during manufacturing or storage, oxidation can occur resulting in the formation of minor alkaloids (Kisaki et al., 1978).

**Figure 3: Relative concentrations of minor alkaloids in e-liquids.**

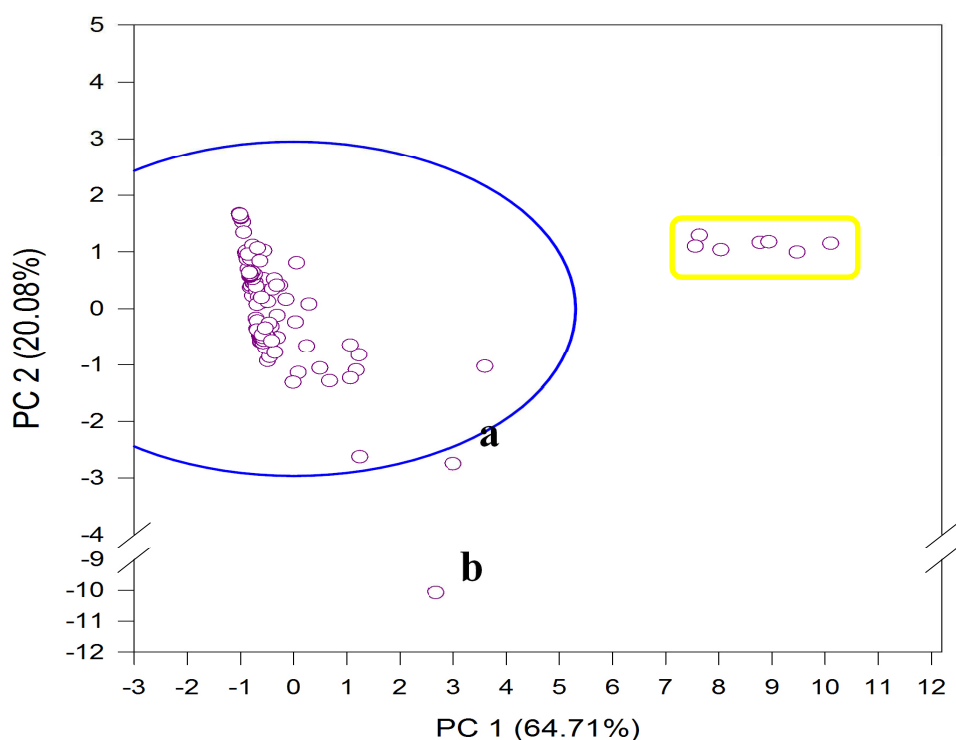


Principal components analysis (PCA), a multivariate statistical technique, was carried out to further investigate the obtained results (Figure 4).

The blue ellipse (only partially displayed in Figure 4) indicates the 95% confidence interval. With high probability, points outside the blue ellipse do not belong to the group of points (e-liquid test samples) within the ellipse. The two points below the ellipse represent a sample that was wrongly labelled (labelled as zero nicotine, but containing about 13.6 mg NIC per millilitre liquid), and the sample with the highest measured NIC content (46.4 mg/ml) among all tested samples. The points within the yellow rectangle represent extracts of cigarette tobacco. The graphical separation of the group of nicotine-containing e-liquids from tobacco extracts allows concluding that it is possible to discriminate e-liquids and tobacco products based on their tobacco alkaloid profile. In turn, it demonstrates also that it is not possible to define characterising elements of e-liquids solely via their tobacco alkaloid profiles. This outcome could be expected as most producers would use NIC complying with USP or Eur. Ph. specifications for the preparation of e-liquids. Particularly NIC needs to be of at least 99% purity, whereas the relative NIC content of cigarette tobacco in the current study was between 92.9% and 94.2% of total tobacco alkaloids, which agrees well with the 95% reported by Sisson and Severson (1990). The small difference might be explained by both the larger number of tobacco alkaloids determined in the current study, which contributed around 1% to the total tobacco alkaloid content, and slight differences in the extraction protocols.

**Figure 4: Principle component analysis – Score plot of PC1 and PC2 for nicotine containing e-liquids and cigarette tobacco extracts**

(a, mislabelled sample; b, sample with the highest measured NIC content)



The presence of minor tobacco alkaloids in e-liquids is not sufficient to indicate the source of NIC (natural or synthetic), and therefore identify the product as a tobacco product, as specified in the TPD (EU, 2014). Synthetic NIC, which is offered on the market specifically for the preparation of e-liquids, might, despite high purity claims, also contain minor tobacco alkaloids, most likely intermediates of the synthesis ("TFN@ Nicotine," 2016). A possibility to distinguish NIC derived from *Nicotiana tabaccum* from synthesized NIC might be offered by chiral chromatography, as natural NIC occurs primarily as (-)-(S)-stereoisomer, and chemically synthesized NIC is likely to be a racemic mixture of (-)-(S)- and (-)-(R)-forms. This option would need, however, further investigations.

## **Volatile profiles of tobacco and non-tobacco flavoured e-liquids**

The aim of the evaluation of the flavour profiles of e-liquids was to identify possibilities to discriminate tobacco-derived flavours from non-tobacco flavours. For that purpose, different e-liquids were analysed by gas chromatography - high-resolution mass spectrometry for their volatile and semi-volatile constituents.

The NIST mass spectral library was used for peak identification. Additionally, Linear Retention Indices (LRIs) were calculated for each peak, and compared to literature data retrieved from several online databases. Finally, literature was searched in order to evaluate whether the particular substance was already identified as genuine tobacco constituent (Rodgman & Perfetti, 2013). This resulted in a set of 90 substances, which corresponded to about 90 % of all chemical compounds found in the different e-liquids. Details of the analysed substances are provided in Annex 2 in Table A-5. The number of substances is rather small compared to natural tobacco, in which more than 5000 volatile substances were identified so far (Perfetti & Rodgman, 2011). However, the difference can be explained by the influence of the type of tobacco used for the preparation of the flavour extract, the extraction procedure and solvent used, and a potential further fractionation of the obtained extract. The low concentration of flavourings in the e-liquid, which is about 1 % of the total volume, additionally impedes the detectability of minor flavour constituents.

## 1.2 Samples

E-liquid samples were categorized based on the declared flavour into the following categories: tobacco, coffee, vanilla, menthol, fruit, and other flavours. In total, 108 e-liquids were analysed. About 40 % of the analysed samples contained tobacco flavours according to the label declaration (Table 2). Details of the individual samples analysed are presented in Annex 2 in Table A-4.

**Table 2: Number of test samples per flavour category**

<b>Flavour</b>	<b>Number of samples</b>
Tobacco	43
Coffee	8
Vanilla	8
Mint	9
Fruit	29
Other	11

### 1.2.1 Sample preparation and analysis

The sample preparation was designed with a view to minimise potential sample discrimination during sample preparation. Aliquots of the test samples were only diluted in methanol, fortified by the stable isotope labelled internal standard, and introduced into the gas chromatograph hyphenated to a QTOF high resolution mass spectrometer. Sample injection occurred via a thermal desorption inlet system. Chromatograms were recorded in total ion scan mode over the mass-to-charge range of 45 to 450. The internal standard was used to monitor fluctuations of the instrument sensitivity. As they were rather low, absolute signal abundances (peak areas) were used for data evaluation. Annex 2 provides the list of identified substances, the selection and classification of test samples as well as details of the applied analytical method.

## 1.3 Data Analysis

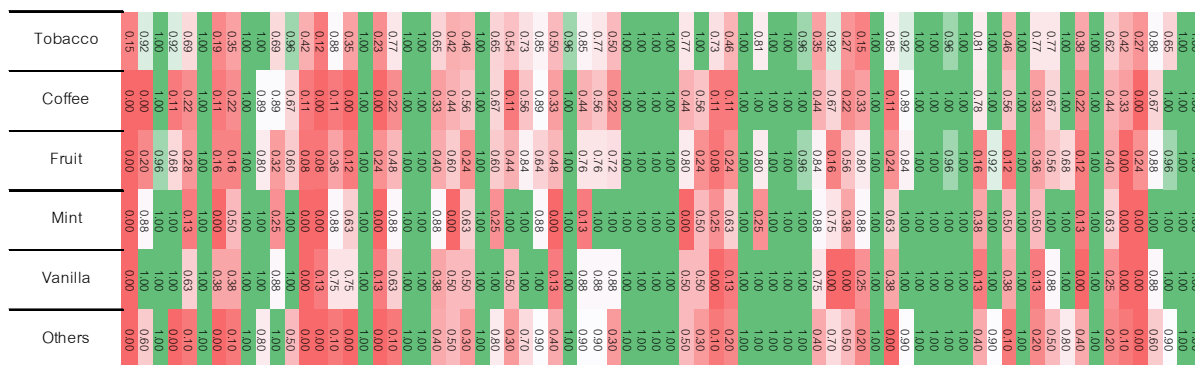
Data analysis was based on a data matrix with about 9700 entries.

The first step concerned the identification of volatiles with high discriminative power with the aim to reduce the number of analytes for model building. The second step concerned the development of a model for the flavour based discrimination of tobacco-flavoured e-liquids from non-tobacco-flavoured e-liquids.

### 1.3.1 Classification of samples

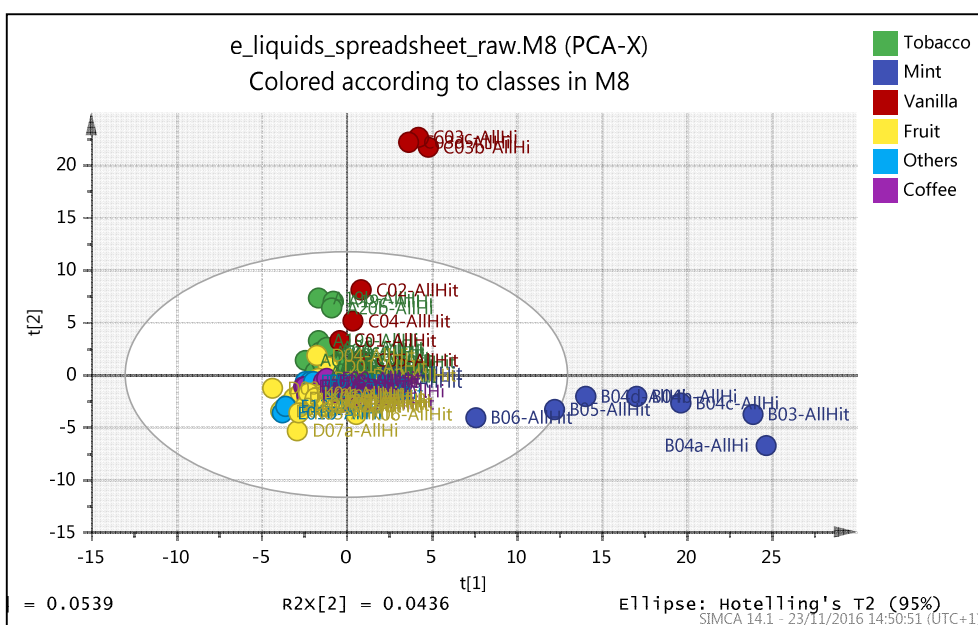
The volatile composition of e-liquids is complex and not uniform at all. They contain typically a large number of different substances at varying concentration levels. Several volatile substances occurring in tobacco extracts are also constituents of e-liquids belonging to other non-tobacco flavoured categories. Figure 5 displays a map of the relative prevalence of the measured substances in the differently flavoured e-liquids. Green colour indicates the presence of the particular substance in all tested samples of this e-liquid category, whereas red colour means the opposite. Pale colours point to relative prevalence in-between the two extremes.

Figure 5: Prevalence of measured substances in different e-liquid categories



Principle component analysis of the whole data set was employed for elucidating whether there is a significant difference in the composition of e-liquids. Figure 6 shows a graph of the first two principle components. Only menthol flavoured e-liquids and some vanilla flavoured e-liquids could be distinguished from the bulk of samples.

Figure 6: PCA score plot of e-liquid volatiles:





The prediction of whether a particular e-liquid contains a tobacco extract is not simple, as there is not a single identifier for tobacco extracts, and many substances measured in tobacco extracts also occur in other plant extracts. Hence, the overlap of the chemical profiles of different plant based flavourings, together with their variability in composition prevents the application of simple mathematical models. A number of multivariate statistical methods/algorithms, i.e. partial least square discrimination analysis (PLS-DA) and decision tree (DT) prediction, were employed for class prediction.

### 1.3.1.1 Partial least square discrimination

PLS-DA aims to find relations between the measured signals (raw data matrix) and the classification matrix (flavour category). Two different levels of classification were tested. One comprised five flavour classes (tobacco, mint, vanilla, fruit, and others) while the other distinguished only between tobacco and non-tobacco flavour. Each model was set up with the same 79 trainings samples, and only those volatile substances with high discrimination power were used for fitting. The developed models were validated both against a subset of the training samples (cross-validation) as well as against independent test samples.

For cross-validation of the developed statistical models the 79 samples were split in three groups. Two of the three groups were used to build the PLS-DA model. The model is then used to classify the samples in the third group, and results are evaluated for their agreement with the known class membership of samples. This procedure is ten times repeated, each time with random allocation of the samples to different groups. Table 3 presents the outcome of the cross-validation of the PLS-DA models.

**Table 3: Cross-validation of the PLS-DA model based on five flavour groups**

	[Fruit] (Predict...	[Mint] (Predict...	[Others] (Predi...	[Tobacco] (Pre...	[Vanilla] (Predi...	Accuracy
(True) [Fruit]	16	0	3	1	0	80.000
(True) [Mint]	0	8	0	0	0	100.000
(True) [Others]	3	1	14	2	0	70.000
(True) [Tobacco]	0	0	2	24	0	92.308
(True) [Vanilla]	0	0	0	0	5	100.000
Overall Accuracy						84.810

The accuracy (percentage of correct classifications) was 92 % for tobacco-flavoured samples about, whereas the overall accuracy reached about 85 %.

The samples highlighted in Table 4 in green represent tobacco-flavoured e-liquids, whereas all others correspond to different flavours. Of the 21 independent validation samples, three out of the ten tobacco flavoured samples and two samples with other flavours were misclassified (red ellipses). Challenges for correct classification are provided by samples that contain next to tobacco another characteristic flavour, e.g. sample F03, which was labelled by the producer as "shisha coffee", contains besides tobacco also a coffee flavouring. The same was true for sample C02, which was labelled as "shisha vanilla". Despite these two class-overlapping samples, the accuracy of classification agrees well with the outcome of cross validation.

**Table 4: Individual results from validation of the PLS-DA model based on five flavour groups with independent samples**

Sample Name	Predicted	Confidence Measure
A03-AllHits	[Tobacco]	0.675
A06b-AllHits	[Tobacco]	0.573
A09-AllHits	[Tobacco]	0.711
A19a-AllHits	[Tobacco]	0.526
A23-AllHits	[Tobacco]	0.413
A26-AllHits	[Tobacco]	0.653
A30-AllHits	[Mint]	0.352
A31-AllHits	[Tobacco]	0.607
B03-AllHits	[Mint]	0.609
C02-AllHits	[Vanilla]	0.428
C05-AllHits	[Mint]	0.323
D02-AllHits	[Mint]	0.603
D07a-AllHits	[Fruit]	0.474
D08b-AllHits	[Fruit]	0.502
E03-AllHits	[Others]	0.479
E06-AllHits	[Others]	0.551
E14-AllHits	[Others]	0.408
F03-AllHits	[Others]	0.559
F05-AllHits	[Others]	0.649
K03-AllHits	[Fruit]	0.467
K09-AllHits	[Fruit]	0.443

Flavour identifier: A: tobacco; B: mint; C: vanilla; D and K: fruit; E and F: others. green highlighted: contains tobacco flavour; red ellipses indicate wrong classifications

Reducing the number of classes to tobacco-flavoured and non-tobacco flavoured (binary classification), and restricting the model only to parameters of high significance ( $p > 0.05$ ) provided similar overall accuracy of classification, but improved accuracy in identification of tobacco-flavoured e-liquids. Table 5 contains accuracy data obtained by cross validation of the model according to the method described earlier.

**Table 5: Cross-validation of the PLS-DA model based on two flavour groups**

	[Non-to...	[Tobacco...	Accuracy
(True) [Non-tobacco]	41	12	77.358
(True) [Tobacco]	0	26	100.000
Overall Accuracy			84.810

Validation of the developed model with independent samples led to very promising results. All thirty samples were correctly classified (Table 6).

**Table 6: Individual results from validation of the PLS-DA model based on two flavour groups with independent samples**

Sample Name	Predicted	Confidence Measure
A03-AllHits	[Tobacco]	1.000
A06b-AllHits	[Tobacco]	1.000
A09-AllHits	[Tobacco]	1.000
A19a-AllHits	[Tobacco]	1.000
A23-AllHits	[Tobacco]	1.000
A26-AllHits	[Tobacco]	1.000
A30-AllHits	[Tobacco]	1.000
A31-AllHits	[Tobacco]	1.000
A32-AllHits	[Tobacco]	1.000
A33-AllHits	[Tobacco]	1.000
A34-AllHits	[Tobacco]	1.000
A35-AllHits	[Tobacco]	1.000
A36-AllHits	[Tobacco]	1.000
A37-AllHits	[Tobacco]	1.000
B03-AllHits	[Non-tobacco]	1.000
B07-AllHits	[Tobacco]	1.000
C02-AllHits	[Tobacco]	1.000
C05-AllHits	[Non-tobacco]	1.000
D02-AllHits	[Non-tobacco]	1.000
D07a-AllHits	[Non-tobacco]	1.000
D08b-AllHits	[Non-tobacco]	1.000
E03-AllHits	[Non-tobacco]	1.000
E06-AllHits	[Non-tobacco]	1.000
E14-AllHits	[Non-tobacco]	1.000
F15-AllHits	[Tobacco]	1.000
F03-AllHits	[Tobacco]	1.000
F05-AllHits	[Non-tobacco]	1.000
F10-AllHits	[Tobacco]	1.000
K03-AllHits	[Non-tobacco]	1.000
K09-AllHits	[Non-tobacco]	1.000

Green highlighted cell: samples containing tobacco flavour

### 1.3.1.2 Decision tree

Decision tree classification is an analysis method used in data mining of large data collections. It builds classification models in the form of a tree structure, thereby breaking down the input data set into smaller subsets. Cross validation was performed using the method specified earlier. The overall accuracy was less favourable than with PLS-DA (Table 7). Especially the correct classification rate of tobacco containing e-liquids was in cross-validation lower compared to PLS-DA.

**Table 7: Accuracy table for cross validation of decision tree model**

	[Non-to...	[Tobacco...	Accuracy
(True) [Non-tobacco]	46	7	86.792
(True) [Tobacco]	8	18	69.231
Overall Accuracy			81.013

However, the validation with independent samples revealed a high level of correct classifications (Table 8). Two out of 16 tobacco-flavoured samples were wrongly classified (red ellipses). However, sample A30 contains besides tobacco flavour significant amounts of menthol, which might cause the misclassification.

**Table 8: Validation of decision tree model with independent samples**

Sample Name	Predicted	Confidence Measure
A03-AIHHits	(Non-tobacco)	0.075
A06b-AIHHits	[Tobacco]	0.231
A09-AIHHits	[Tobacco]	0.115
A19a-AIHHits	[Tobacco]	0.577
A23-AIHHits	[Tobacco]	0.577
A26-AIHHits	[Tobacco]	0.577
A30-AIHHits	(Non-tobacco)	0.849
A31-AIHHits	[Tobacco]	0.577
A32-AIHHits	[Tobacco]	0.577
A33-AIHHits	[Tobacco]	0.577
A34-AIHHits	[Tobacco]	0.577
A35-AIHHits	[Tobacco]	0.577
A36-AIHHits	[Tobacco]	0.577
A37-AIHHits	[Tobacco]	0.577
B03-AIHHits	[Non-tobacco]	0.849
B07-AIHHits	[Non-tobacco]	0.849
C02-AIHHits	[Non-tobacco]	0.038
C05-AIHHits	[Non-tobacco]	0.849
D02-AIHHits	[Non-tobacco]	0.849
D07a-AIHHits	[Non-tobacco]	0.849
D08b-AIHHits	[Non-tobacco]	0.849
E03-AIHHits	[Non-tobacco]	0.075
E06-AIHHits	[Non-tobacco]	0.849
E14-AIHHits	(Tobacco)	0.577
E15-AIHHits	[Tobacco]	0.577
F03-AIHHits	[Non-tobacco]	0.075
F05-AIHHits	[Non-tobacco]	0.075
F10-AIHHits	[Tobacco]	0.577

Green highlighted cell: samples containing tobacco flavour, red ellipses indicate misclassifications

## Conclusion

In agreement with previous literature reports, the measured NIC concentrations of e-liquids were often significantly lower than the concentrations indicated on the package. The data showed that the profiles of minor alkaloids vary considerably among the different samples tested. As most of the NIC currently used to produce e-liquids is obtained from natural tobacco, the minor alkaloid profiles are generally affected by the quality of the purification processes. Based on the obtained results, the analysis of tobacco alkaloids does not seem to be suitable for tracing back the origin of NIC contained in e-liquids to a natural source or made by chemical synthesis. The presence of minor tobacco alkaloids might be an indicator for the natural origin of NIC. However, it cannot be excluded that chemically synthesized NIC contains similar impurities. Additionally, modern purification processes might lead for both natural and synthetic NIC to qualities that do not introduce in the e-liquid detectable levels of other minor tobacco alkaloids.

The flavour profiles of the investigated test samples are complex. The distinction between tobacco-flavoured e-liquids and non-tobacco-flavoured e-liquids is challenging. About 90 substances were identified in tobacco-containing e-liquids. They were systematically analysed in about 110 test samples, of which 70 % were applied for setting-up statistical classification models and about 30 % were used to validate the developed models. Two different multivariate classification methods were applied to the test samples, in particular partial least square discrimination and decision tree classification. Both methodologies provided suitable results, allowing the discrimination of tobacco-flavoured and non-tobacco-flavoured e-liquids with a high level of accuracy. However, partial least square discrimination performed slightly better. Samples containing both tobacco flavours and other characteristic flavours challenge both models. Including new training samples in the models of e.g. mixed flavours and, if applicable, of new flavours should be envisaged in order to maintain their discrimination power.

# ANNEX 1 – Analytical method for the determination of tobacco alkaloids in e-liquids, test samples, and results

## A1.1. Analysis of tobacco alkaloids by HPLC-MS/MS in HILIC mode

### 1. Standards and reagents

Following reference substances were acquired from Sigma-Aldrich, Diegem, Belgium. Information on purity, concentration and solvent are given within brackets.

- (1) (+)-anabasine hydrochloride (1.000 ± 0.005 mg/mL in methanol)
- (2) (–)-cotinine (1.000 ± 0.005 mg/mL in methanol)
- (3) Cytisine (≥99%)
- (4) 4,4'-dipyridyl (98%)
- (5) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (1.000 ± 0.005 mg/mL in methanol)
- (6) 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (≥98%)
- (7) Myosmine (≥98%)
- (8) (–)-nicotine (1.00 ± 0.05 mg/mL in methanol)
- (9) N-nitrosoanabasine (≥99%)
- (10) N-nitrosoanatabine (≥97%)
- (11) (±)-N-nitrosornnicotine (1.000 ± 0.005 mg/mL in methanol)
- (12) (R,S)-norcotinine (1.000 ± 0.005 mg/mL in methanol)
- (13) (±)-nornicotine (1.000 ± 0.005 mg/mL in methanol)
- (14) (±)-cotinine-D<sub>3</sub> (1.0 mg/mL in methanol)
- (15) (±)-nicotine-D<sub>4</sub> (100 µg/mL in acetonitrile)
- (16) (±)-nornicotine-D<sub>4</sub> (100 µg/mL in methanol)
- (17) Ammonium acetate (NH<sub>4</sub>Ac) LCMS grade from Sigma-Aldrich
- (18) Acetonitrile LCMS grade

(R,S)-anatabine (≥95%) was purchased at Cayman Chemical, Ann Arbor, Michigan, USA.

Mixed standard solution of stable isotope labelled internal standards (IS):

Mix IS	Conc. (µg/mL)
NIC-d4	5.0622
NNIC-d4	0.8943
COT-d3	0.3130

Calibration solutions for tobacco alkaloids:

NIC: 20 ng/mL- 2000 ng/mL

Minor tobacco alkaloids: 10 ng/mL-1000 ng/mL

## **2. Methods**

### **2.1. Sample preparation**

The relative proportions of nicotine content compared to the contents of minor tobacco alkaloids required two separated assays per test sample. Two dilutions were prepared of each tested e-liquid, in order to bring the analyte concentrations into the linear range of the HPLC-MS/MS instrument. Cigarette tobacco was initially extracted with aqueous methanol. The extract was further diluted according to the scheme of dilutions of e-liquids.

A) Determination of minor tobacco alkaloids: low dilution (20-fold dilution) of e-liquid:

- (1) Add 100  $\mu$ L e-liquid sample to 1.900 mL ACN/water (1:1) in a 2 mL Eppendorf vial and vortex
- (2) Pipette 0.5 mL dilution into a HPLC vial, add 100  $\mu$ L IS solution and homogenate on a Vortex mixer.
- (3) Filtrate through 0.20  $\mu$ m regenerated cellulose filter syringe filter

B) Determination of nicotine: high dilution (20.000- fold dilution):

1. Add 100  $\mu$ L e-liquid sample to 1.900 mL ACN/water (1:1) in a 2 mL Eppendorf vial and vortex
2. Pipette 10  $\mu$ L dilution into 10 mL ACN/water (1:1) in a 15 mL centrifuge plastic tube
3. Pipette 0.5 mL dilution into a HPLC vial, add 100  $\mu$ L IS solution and vortex
4. Filtrate through 0.20  $\mu$ m regenerated cellulose filter syringe filter

C) Cigarettes

5. Add 10 mL MeOH/water (80:20, v/v) to the tobacco content of 1 cigarette (aprox. 500 mg) in a 15 mL centrifuge plastic tube
6. Ultrasound extraction for 30 min
7. Centrifuge at 3500 RPM 5 min and collect supernatant
8. Follow same dilution scheme as for the e-liquids

## 2.2. HPLC-MS/MS analysis

### HPLC Column:

Ascentis Express F5 (10 cm x 2.1 mm, 2.7  $\mu$ m) from Supelco

Guard column Discovery Ascentis Express F5 (0.5 cm x 2.1 mm, 2.7  $\mu$ m)

Mobile Phase A: 5 mM aqueous NH<sub>4</sub>Ac

Mobile Phase B: acetonitrile

Mobile phase flow: 0.500 ml/min

Column temperature: 45 °C

Injection volume 0.5  $\mu$ L

### Gradient elution program:

Time (min)	% B
0.50	95
3.00	80
6.00	70
9.50	70
9.51	95
14.50	95

### MS/MS method

Instrument: AB SCIEX™ QTRAP® 6500

### Instrument parameters:

CUR:	30
CAD:	Medium
IS: Positive	4500
TEM	500
GS1	70
GS2	50
DP	20
EP	10
ST1	-20



Acquisition in two time segments/periods:

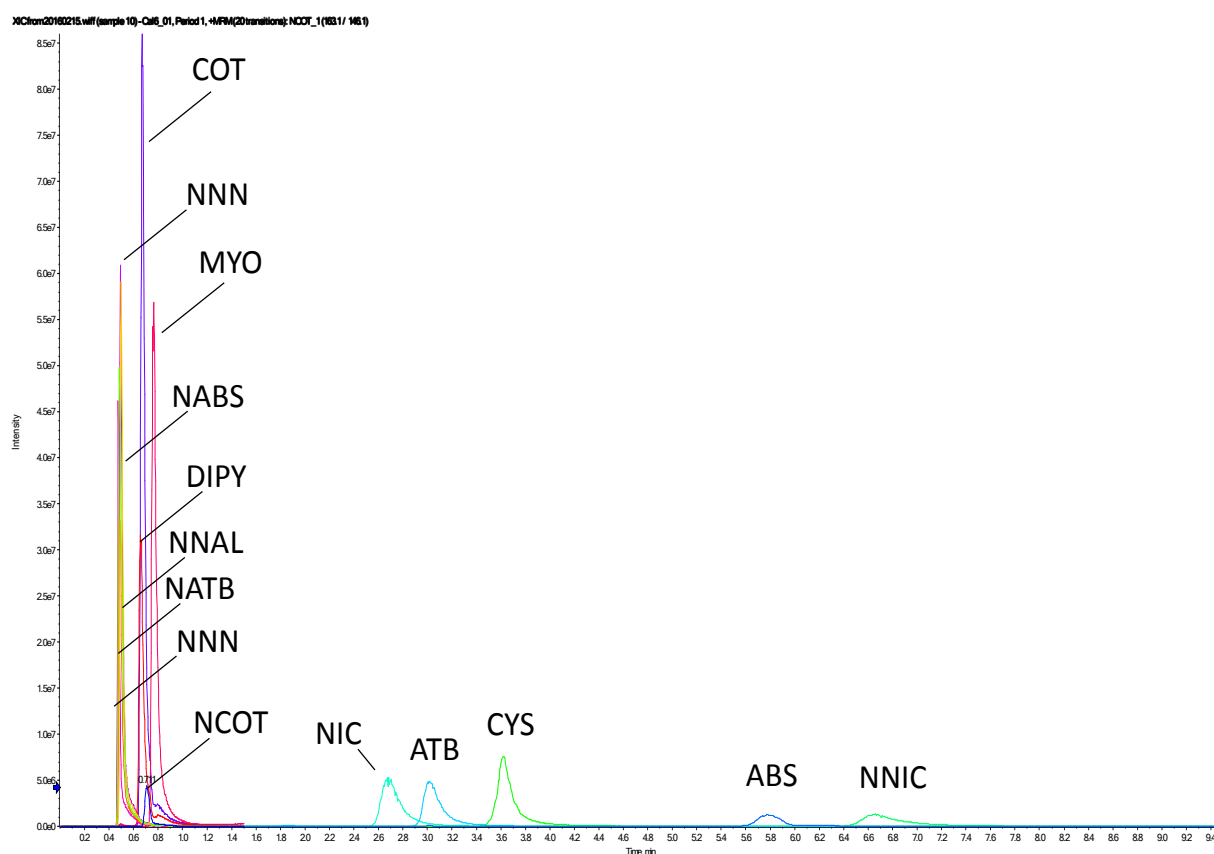
Period 1 (weakly retained compounds)

Q1 Mass (m/z)	Q3 Mass (m/z)	Dwell time (msec)	CE (ev)	CXP (V)	ID
163.1	146.1	5	15	12	NCOT_1
163.1	135.1	5	30	12	NCOT_2
177.1	80.0	5	30	12	COT_1
177.1	98.0	5	28	12	COT_2
180.1	80.0	5	30	12	COT-d3_1
180.1	101.0	5	28	12	COT-d3_2
178.1	148.1	5	15	4	NNN_1
178.1	120.1	5	25	10	NNN_2
208.1	122.0	5	15	14	NNK_1
208.1	106.0	5	29	14	NNK_2
147.0	105.0	5	31	12	MYO_1
147.0	129.9	5	29	14	MYO_2
157.0	77.0	5	45	12	DIPY_1
157.0	130.1	5	35	8	DIPY_2
190.1	160.1	5	13	12	NATB_1
190.1	79.1	5	37	12	NATB_2
192.1	162.1	5	15	12	NABS_1
192.1	133.1	5	29	8	NABS_2
210.1	93.0	5	31	18	NNAL_1
210.1	149.0	5	19	16	NNAL_2

Period 2 (stronger retained compounds)

Q1 Mass (m/z)	Q3 Mass (m/z)	Dwell time (msec)	CE (ev)	CXP (V)	ID
191.1	147.9	20	27	16	CYS_1
191.1	133	20	43	16	CYS_2
149.1	132.1	20	17	6	NNIC_1
149.1	80.0	20	27	10	NNIC_2
153.1	84.0	20	27	10	NNIC-d4_1
153.1	110.1	20	23	10	NNIC-d4_2
163.1	132.1	20	20	12	NIC_1
163.1	106.1	20	22	12	NIC_2
167.1	136.0	20	20	12	NIC-d4_1
167.1	84.0	20	25	12	NIC-d4_2
161.0	144.0	20	28	12	ATB_1
161.0	80.0	20	32	10	ATB_2
163.1	94.1	20	25	12	ABS_1
163.1	146	20	20	12	ABS_2

Figure 1. MRM chromatograms for a calibration standard containing all target compounds



### Legend

NIC	Nicotine	DIPY	4,4'-Dipyridyl
NNIC	Nornicotine	NNN	N-Nitrosornicotine
ATB	Anatabine	NATB	N-Nitrosoanatabine
ABS	Anabasine	NABS	N-Nitrosoanabasine
MYO	Myosmine	NNAL	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol
COT	Cotinine	NNK	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone
NCOT	Norcotinine	CYS	Cytisine

## A1.2. Test samples

Table A- 1: E-liquid samples analysed for tobacco alkaloid contents

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
001	Tobacco	UK	PRC	Tobacco	0
002	Smooth	UK	PRC	Tobacco	0
003	Menthol	UK	PRC	Fresh	0
004	Blueberry	UK	PRC	Fruity	0
005	Smooth	UK	PRC	Tobacco	6
006	Rainbow	UK	PRC	Tobacco	12
007	Apple	UK	PRC	Fruity	18
008	Virginia Tobacco	UK	PRC	Tobacco	18
009	Blueberry	UK	PRC	Fruity	18
010	Tobacco	UK	PRC	Tobacco	18
011	Strawberry	UK	PRC	Fruity	18
012	Cherry	UK	PRC	Fruity	24
013	Cherry Limeade	DE	USA	Fruity	0
014	Calamity Jane Tobacco	DE	USA	Tobacco	0
015	Ol' River Tobacco	DE	USA	Tobacco	0
016	Rocket Blend	DE	USA	Sweet	0
017	Calamity Jane Tobacco	DE	USA	Tobacco	12
018	Cherry Limeade	DE	USA	Fruity	12
019	Ol' River Tobacco	DE	USA	Tobacco	18
020	Cherry Limeade	DE	USA	Fruity	18
021	Calamity Jane Tobacco	DE	USA	Tobacco	18
022	Coffee	DE	ES	Non-alcoholic drink	0
023	Coffee	DE	ES	Non-alcoholic drink	6
024	Coffee	DE	ES	Non-alcoholic drink	16
025	Tobacco	DE	DE	Tobacco	0

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
026	Black Mamba	DE	DE	Tobacco	0
027	Black Fire	DE	DE	Tobacco	0
028	Pfeife	DE	DE	Tobacco	0
029	Kentucky	DE	DE	Tobacco	0
030	American Blend	DE	DE	Tobacco	0
031	Maxx Blend	DE	DE	Tobacco	0
032	Dessert	DE	DE	Tobacco	0
033	7 leaves	DE	DE	Tobacco	0
034	RY4	DE	DE	Tobacco	0
035	Toffee	DE	DE	Sweet	0
036	Karamell Sahne	DE	DE	Sweet	0
037	After Nine	DE	DE	Sweet	0
038	Marzipan	DE	DE	Sweet	0
039	Ice Bombon	DE	DE	Sweet	0
040	Gummi-barchen	DE	DE	Sweet	0
041	Kase-kuchen	DE	DE	Sweet	0
042	Apfel-kuchen	DE	DE	Sweet	0
043	Honig	DE	DE	Sweet	0
044	Cookie	DE	DE	Sweet	0
045	Zitrone fresh	DE	DE	Fresh	0
046	Kirsch Amarena fresh	DE	DE	Fresh	0
047	Erdbeer fresh	DE	DE	Fresh	0
048	Walderdbeer fresh	DE	DE	Fresh	0
049	Apfel fresh	DE	DE	Fresh	0
050	Brombeer fresh	DE	DE	Fresh	0
051	Kiba fresh	DE	DE	Fresh	0
052	Pfirsich Maracuja fresh	DE	DE	Fresh	0
053	Himbeer fresh	DE	DE	Fresh	0
054	Wassermelone fresh	DE	DE	Fresh	0

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
055	Pfirsich Maracuja	DE	DE	Fruity	10
056	Zitrone	DE	DE	Fruity	10
057	Honigmelone	DE	DE	Fruity	10
058	Kiba	DE	DE	Fruity	10
059	Himbeer	DE	DE	Fruity	10
060	Frucht-mix Tropic	DE	DE	Fruity	10
061	Erdbeer	DE	DE	Fruity	10
062	Cocos	DE	DE	Fruity	10
063	Apfel	DE	DE	Fruity	10
064	Amarena kirsch	DE	DE	Fruity	10
065	Tobacco	DE	DE	Tobacco	10
066	Black Mamba	DE	DE	Tobacco	10
067	Black Fire	DE	DE	Tobacco	10
068	Pfeife	DE	DE	Tobacco	10
069	Kentucky	DE	DE	Tobacco	10
070	American Blend	DE	DE	Tobacco	10
071	Maxx Blend	DE	DE	Tobacco	10
072	Dessert	DE	DE	Tobacco	10
073	7 leaves	DE	DE	Tobacco	10
074	RY4	DE	DE	Tobacco	10
075	Toffee	DE	DE	Sweet	20
076	Karamell Sahne	DE	DE	Sweet	20
077	After Nine	DE	DE	Sweet	20
078	Marzipan	DE	DE	Sweet	20
079	Ice Bombon	DE	DE	Sweet	20
080	Gummi-barchen	DE	DE	Sweet	20
081	Kase-kuchen	DE	DE	Sweet	20
082	Apfel-kuchen	DE	DE	Sweet	20
083	Honig	DE	DE	Sweet	20

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
084	Cookie	DE	DE	Sweet	20
085	Zitrone fresh	DE	DE	Fresh	20
086	Kirsch Amarena fresh	DE	DE	Fresh	20
087	Erdbeer fresh	DE	DE	Fresh	20
088	Walderdbeer fresh	DE	DE	Fresh	20
089	Apfel fresh	DE	DE	Fresh	20
090	Brombeer fresh	DE	DE	Fresh	20
091	Kiba fresh	DE	DE	Fresh	20
092	Pfirsich Maracuja fresh	DE	DE	Fresh	20
093	Himbeer fresh	DE	DE	Fresh	20
094	Wassermelone fresh	DE	DE	Fresh	20
095	Pfirsich Maracuja	DE	DE	Fruity	20
096	Zitrone	DE	DE	Fruity	20
097	Honigmelone	DE	DE	Fruity	20
098	Kiba	DE	DE	Fruity	20
099	Himbeer	DE	DE	Fruity	20
100	Frucht-mix Tropic	DE	DE	Fruity	20
101	Erdbeer	DE	DE	Fruity	20
102	Cocos	DE	DE	Fruity	20
103	Apfel	DE	DE	Fruity	20
104	Amarena kirsch	DE	DE	Fruity	20
105	Tobacco	DE	DE	Tobacco	20
106	Black Mamba	DE	DE	Tobacco	20
107	Black Fire	DE	DE	Tobacco	20
108	Pfeife	DE	DE	Tobacco	20
109	Kentucky	DE	DE	Tobacco	20
110	American Blend	DE	DE	Tobacco	20
111	Maxx Blend	DE	DE	Tobacco	20
112	Dessert	DE	DE	Tobacco	20

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
113	7 leaves	DE	DE	Tobacco	20
114	RY4	DE	DE	Tobacco	20
115	Et Spiritum	FR	FR	Exotic	11
116	Unknown	-	-	-	-
117	Crisp Mint	UK	-	Fresh	6
119	Unknown	UK	-	-	-
120	Unknown	UK	-	-	-
121	Unknown	UK	-	-	-
122	Unknown	UK	-	-	-
123	Arabic tobacco	NL	NL	Tobacco	0
124	USA Mix	NL	NL	Tobacco	0
125	Turkish blend	NL	NL	Tobacco	0
126	Southeast County	NL	NL	Tobacco	0
127	Cuba Cigar	NL	NL	Tobacco	0
128	Honey Toasted Tobacco	NL	NL	Tobacco	0
129	Old Dry Tobacco	IT	IT	Tobacco	0
130	The Palm	NL	NL	Tobacco	0
131	Coco & Havana	NL	NL	Tobacco	0
132	Coco & Havana	NL	NL	Tobacco	6
133	Coco & Havana	NL	NL	Tobacco	12
134	Coco & Havana	NL	NL	Tobacco	18
135	Tobacco Mint	NL	NL	Fresh	0
136	Menthol	NL	NL	Fresh	0
137	Arctic Mint	NL	NL	Fresh	0
138	Mint	NL	NL	Fresh	0
139	Ice Mint	NL	NL	Fresh	0
140	Ice Mint	NL	NL	Fresh	6
141	Ice Mint	NL	NL	Fresh	12
142	Ice Mint	NL	NL	Fresh	18

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
143	Vanilla	NL	NL	Sweet	0
144	Shisha Vanille	NL	NL	Sweet	0
145	Vanille	FR	FR	Sweet	0
146	Ice Vanille	NL	NL	Sweet	0
147	Vanilla Orange	NL	NL	Sweet	0
148	Vanilla Orange	NL	NL	Sweet	6
149	Vanilla Orange	NL	NL	Sweet	12
150	Vanilla Orange	NL	NL	Sweet	18
151	Passion Fruit	NL	NL	Fruity	0
152	Mandarin	NL	NL	Fruity	0
153	Coconut	NL	NL	Fruity	0
154	Pear	NL	NL	Fruity	0
155	Strawberry	NL	NL	Fruity	0
156	Strawberry	NL	NL	Fruity	6
157	Strawberry	NL	NL	Fruity	12
158	Strawberry	NL	NL	Fruity	18
159	Cotton Candy	NL	NL	Sweet	0
160	White Chocolate	NL	NL	Sweet	0
161	Banana Chocolate	NL	NL	Sweet	0
162	Pumpkin Pie	NL	NL	Sweet	0
163	Biscuit	IT	IT	Sweet	0
164	Banana Split	NL	NL	Sweet	0
165	Banana Split	NL	NL	Sweet	6
166	Banana Split	NL	NL	Sweet	12
167	Banana Split	NL	NL	Sweet	18
168	Cola	NL	NL	Non-alcoholic drink	0
169	Shisha Coffee	NL	NL	Non-alcoholic drink	0
170	Cappuccino	NL	NL	Non-alcoholic drink	0
171	Herbal Tea	NL	NL	Non-alcoholic drink	6



Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
172	Herbal Tea	NL	NL	Non-alcoholic drink	12
173	Caramel Machiato	NL	NL	Non-alcoholic drink	0
174	Caramel Machiato	NL	NL	Non-alcoholic drink	12
175	Caramel Machiato	NL	NL	Non-alcoholic drink	18
176	Beer	NL	NL	Alcoholic drink	0
177	Red Wine	NL	NL	Alcoholic drink	0
178	Kriek	FR	FR	Alcoholic drink	0
179	Champagne	NL	NL	Alcoholic drink	6
180	Tequila	NL	NL	Alcoholic drink	0
181	Tequila	NL	NL	Alcoholic drink	6
182	Tequila	NL	NL	Alcoholic drink	12
183	Tequila	NL	NL	Alcoholic drink	18
184	Exotic	FR	FR	Exotic	0
185	Enjoy	FR	FR	Exotic	0
186	Supreme	FR	FR	Exotic	6
187	Relax	FR	FR	Exotic	0
188	Relax	FR	FR	Exotic	18
189	Jet Fresh	FR	FR	Exotic	0
190	Jet Fresh	FR	FR	Exotic	6
191	Jet Fresh	FR	FR	Exotic	12
192	Thyme	NL	CZ	Fresh	0
193	Sage	NL	CZ	Fresh	0
194	Rosemary	NL	CZ	Fresh	0
195	Arctic Mint	BE	-	Fresh	16
196	Tobacco zero	DE	-	Tobacco	0
197	Nevada tobacco	DE	-	Tobacco	18
198	Mint	FI	-	-	0
199	Tiramisu	FI	DE	Sweet	0
200	Cheese cake	CR	CR	Sweet	0

Code	Description	Country acquisition	Country production	Flavour classification	Nicotine content declared (mg/mL)
201	Tobacco natural	CR	CR	Tobacco	48
202	Cappuccino	CR	CR	Non-alcoholic drink	18
203	Marbi	HU	HU	Tobacco	12
204	Real tobacco	HU	HU	Tobacco	0
205	LOP	IT	IT	Sweet	0
206	Venere	IT	IT	Exotic	4
207	Natural flavor violet	FR	LT	Fresh	12
208	Energetinio gerimo	LT	LT	Non-alcoholic drink	12
209	Bubblegum	NL	-	Sweet	6
210	Desert ship	NL	FR	Tobacco	12
211	L&M	NL	-	Tobacco	11
212	Apple mint	PL	PRC	Fresh	0
213	Coffee	PL	PRC	Non-alcoholic drink	6
214	Traditional tobacco	RO	CZ	Tobacco	12
215	Vanilka	CZ	CZ	Sweet	8
216	Energy mix	CZ	CZ	Non-alcoholic drink	8

### A1.3. Contents of tobacco alkaloids determined in e-liquid test samples

**Table A- 2: Results for tobacco alkaloids in tested e-liquids, and relative deviation of nicotine content from declared content**

(red highlighted cells: zero-nicotine labelled products containing nicotine at levels above 0.1 mg/ml; orange highlighted cells: nicotine content deviates more than 10% from declared nicotine content)

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
001	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
002	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
003	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
004	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
005	6	5.997	2.320	4.680	1.498	0.000	5.832	7.306	0.0
006	12	11.224	3.657	5.843	0.893	0.000	6.727	10.120	-6.5
007	18	17.971	10.475	15.472	3.995	0.000	10.813	8.276	-0.2
008	18	16.854	8.366	17.367	2.807	0.000	45.529	61.309	-6.4
009	18	18.721	8.366	22.728	3.084	0.000	49.014	64.397	4.0
010	18	17.803	5.553	10.362	3.009	0.000	22.820	30.757	-1.1
011	18	17.886	11.539	13.164	2.886	0.000	46.239	63.746	-0.6
012	24	22.965	3.625	19.444	2.505	0.196	11.127	14.079	-4.3
013	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
014	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	
015	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
016	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
017	12	9.741	4.084	3.502	5.663	0.000	1.248	0.365	-18.8
018	12	8.687	0.288	3.692	0.527	0.000	0.000	0.000	-27.6
019	18	15.287	7.502	4.097	8.484	0.000	1.927	0.633	-15.1
020	18	13.449	0.985	7.403	6.096	0.000	1.815	0.661	-25.3

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
021	18	17.550	1.128	4.597	0.762	0.000	0.000	0.000	-2.5
022	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
023	6	5.142	1.828	5.681	0.373	0.000	8.731	15.370	-14.3
024	16	16.001	1.327	6.913	1.107	0.000	0.000	0.257	0.0
025	0	0.003	0.000	0.000	0.000	0.000	0.000	0.141	
026	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	
027	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
028	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
029	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
030	0	0.004	0.000	0.000	0.000	0.000	0.000	0.000	
031	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
032	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
033	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	
034	0	0.038	0.000	0.000	0.000	0.000	0.000	0.000	
035	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	
036	0	0.002	0.000	0.000	0.000	0.000	0.000	0.000	
037	0	0.038	0.000	0.000	0.000	0.000	0.000	0.000	
038	0	0.003	0.000	0.000	0.000	0.000	0.000	0.000	
039	0	0.009	0.000	0.000	0.000	0.000	0.000	0.000	
040	0	0.004	0.000	0.000	0.000	0.000	0.000	0.000	
041	0	0.132	0.112	0.179	0.000	0.000	0.000	0.000	
042	0	0.174	0.160	0.388	0.000	0.000	0.000	0.000	
043	0	0.002	0.000	0.000	0.000	0.000	0.000	0.000	
044	0	0.014	0.000	0.000	0.000	0.000	0.000	0.000	
045	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
046	0	0.002	0.000	0.000	0.000	0.000	0.000	0.000	
047	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
048	0	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
049	0	0.002	0.000	0.000	0.000	0.000	0.000	0.000	
050	0	0.001	0.000	0.000	0.000	0.000	0.000	0.000	
051	0	0.003	0.000	0.000	0.000	0.000	0.000	0.000	
052	0	0.001	ND	ND	ND	ND	ND	ND	
053	0	0.001	ND	ND	ND	ND	ND	ND	
054	0	ND	ND	ND	ND	ND	ND	ND	
055	10	8.320	1.773	4.297	1.773	ND	ND	ND	-16.8
056	10	8.117	2.525	4.108	2.228	ND	ND	ND	-18.8
057	10	8.633	5.101	4.262	3.888	ND	ND	ND	-13.7
058	10	8.374	1.803	4.287	1.823	ND	ND	ND	-16.3
059	10	8.472	5.508	4.702	5.844	ND	ND	ND	-15.3
060	10	8.668	5.582	4.723	5.952	ND	ND	ND	-13.3
061	10	8.241	3.811	7.794	1.721	ND	0.827	0.505	-17.6
062	10	8.360	2.863	4.182	3.730	ND	ND	ND	-16.4
063	10	8.649	2.651	4.260	2.416	ND	ND	ND	-13.5
064	10	9.339	1.961	4.845	2.102	ND	ND	ND	-6.6
065	10	8.698	1.213	5.171	0.966	ND	ND	0.217	-13.0
066	10	8.723	1.285	3.316	0.922	ND	ND	ND	-12.8
067	10	8.361	1.237	3.251	0.839	ND	ND	ND	-16.4
068	10	9.005	0.987	4.382	0.999	ND	ND	ND	-9.9
069	10	8.587	0.969	4.282	0.990	ND	ND	ND	-14.1
070	10	8.773	1.323	3.828	1.018	ND	ND	ND	-12.3

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
071	10	8.639	0.935	4.127	0.898	ND	ND	ND	-13.6
072	10	8.498	1.140	3.846	0.880	ND	ND	ND	-15.0
073	10	8.700	1.052	3.414	0.944	ND	ND	ND	-13.0
074	10	9.020	1.183	3.190	0.821	ND	ND	ND	-9.8
075	20	19.169	1.328	8.286	1.675	ND	ND	ND	-4.2
076	20	17.674	0.834	9.785	1.601	ND	ND	ND	-11.6
077	20	17.710	1.199	7.108	1.610	ND	ND	ND	-11.4
078	20	18.391	1.266	10.417	1.551	ND	ND	ND	-8.0
079	20	17.727	1.192	8.247	1.787	ND	ND	ND	-11.4
080	20	17.182	1.402	7.593	1.630	ND	ND	ND	-14.1
081	20	16.895	2.952	9.138	2.125	ND	ND	ND	-15.5
082	20	14.503	5.512	7.730	3.257	ND	1.520	1.124	-27.5
083	20	17.672	1.195	8.087	1.918	ND	ND	ND	-11.6
084	20	17.450	1.638	8.108	1.883	ND	ND	ND	-12.7
085	20	16.561	4.144	7.648	3.087	ND	ND	ND	-17.2
086	20	17.247	1.249	8.270	1.737	ND	ND	ND	-13.8
087	20	17.144	6.323	11.532	2.113	ND	1.750	0.706	-14.3
088	20	18.442	1.512	7.006	1.938	ND	ND	ND	-7.8
089	20	17.911	6.079	5.626	1.529	ND	1.568	0.562	-10.4
090	20	15.009	5.245	17.228	1.715	ND	2.061	0.876	-25.0
091	20	17.828	1.439	6.224	1.568	ND	ND	ND	-10.9
092	20	18.339	1.478	5.741	1.957	ND	ND	ND	-8.3
093	20	18.155	1.241	5.113	1.842	ND	ND	ND	-9.2
094	20	19.352	1.223	5.774	1.867	ND	ND	ND	-3.2
095	20	17.940	1.468	5.735	2.193	ND	ND	ND	-10.3

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
096	20	17.037	3.538	5.377	3.335	ND	ND	ND	-14.8
097	20	16.997	3.478	5.901	2.345	ND	ND	ND	-15.0
098	20	18.552	1.364	5.576	2.130	ND	ND	ND	-7.2
099	20	17.984	1.385	4.720	2.453	ND	ND	ND	-10.1
100	20	17.188	1.860	5.381	2.602	ND	ND	ND	-14.1
101	20	18.460	2.524	7.268	3.226	ND	ND	ND	-7.7
102	20	17.768	1.507	5.452	2.377	ND	ND	ND	-11.2
103	20	16.990	2.562	4.750	2.884	ND	ND	ND	-15.1
104	20	18.321	1.285	5.582	2.632	ND	ND	ND	-8.4
105	20	17.757	2.457	6.354	3.107	ND	ND	ND	-11.2
106	20	16.982	3.003	4.295	4.155	ND	ND	ND	-15.1
107	20	17.938	2.903	3.991	3.664	ND	ND	ND	-10.3
108	20	17.722	2.373	5.327	4.777	ND	ND	ND	-11.4
109	20	19.086	2.553	5.532	5.402	ND	ND	ND	-4.6
110	20	17.257	3.230	4.452	4.451	ND	ND	ND	-13.7
111	20	17.061	2.365	4.975	4.315	ND	ND	ND	-14.7
112	20	17.910	2.541	5.203	4.424	ND	ND	ND	-10.4
113	20	17.384	2.736	4.485	5.129	ND	ND	ND	-13.1
114	20	16.684	2.869	4.197	3.905	ND	ND	ND	-16.6
115	11	9.941	2.653	4.683	2.807	ND	1.012	0.190	-9.6
116	-	17.749	11.932	15.736	12.427	0.271	20.904	76.210	
117	6	4.983	5.986	3.632	12.337	0.193	0.290	0.259	-17.0
119	-	22.139	5.606	221.744	81.624	1.608	6.164	0.690	
120	-	22.416	17.663	16.016	41.067	0.621	1.773	1.271	
121	-	16.027	4.850	11.178	7.142	ND	1.390	0.951	

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
122	-	14.728	3.990	7.926	4.421	ND	1.181	0.798	
123	0	ND	0.143	0.155	ND	ND	0.068	0.024	
124	0	ND	0.175	0.155	ND	ND	0.062	0.003	
125	0	0.390	0.372	0.314	0.295	ND	0.199	0.263	
126	0	0.159	0.179	0.206	0.103	ND	0.096	0.120	
127	0	0.001	0.126	0.156	ND	ND	0.065	0.058	
128	0	0.004	0.125	0.179	ND	ND	0.076	0.428	
129	0	0.028	0.134	0.171	ND	ND	0.113	0.058	
130	0	ND	0.128	0.158	ND	ND	0.065	ND	
131	0	0.009	0.131	0.258	ND	ND	0.090	0.635	
132	6	5.719	0.640	0.786	ND	ND	4.200	22.499	-4.7
133	12	11.639	1.345	1.385	0.031	ND	8.187	47.750	-3.0
134	18	17.560	1.941	1.931	0.070	ND	11.983	72.391	-2.4
135	0	0.001	0.143	0.158	ND	ND	0.071	0.022	
136	0	ND	0.132	0.157	ND	ND	0.061	0.001	
137	0	ND	0.117	0.160	ND	ND	0.067	0.015	
138	0	ND	0.143	0.170	ND	ND	0.075	0.007	
139	0	ND	0.127	0.158	ND	ND	0.064	ND	
140	6	5.636	0.769	0.672	0.084	ND	0.338	11.097	-6.1
141	12	10.927	2.440	3.412	0.229	ND	1.590	78.625	-8.9
142	18	16.848	2.191	2.093	0.143	ND	1.150	40.499	-6.4
143	0	0.001	0.125	0.161	ND	ND	0.067	ND	
144	0	ND	0.154	0.158	ND	ND	0.063	ND	
145	0	ND	0.118	0.158	ND	ND	0.067	0.006	
146	0	ND	0.134	0.162	ND	ND	0.066	0.001	



Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
147	0	ND	0.127	0.157	ND	ND	0.061	0.002	
148	6	6.158	0.967	0.266	ND	ND	0.472	14.733	2.6
149	12	11.983	1.659	0.360	0.035	ND	0.998	33.977	-0.1
150	18	18.390	2.733	0.512	0.071	ND	1.827	52.317	2.2
151	0	0.001	0.121	0.164	ND	ND	0.067	ND	
152	0	ND	0.116	0.163	ND	ND	0.066	ND	
153	0	ND	0.138	0.156	ND	ND	0.064	0.006	
154	0	ND	0.134	0.158	ND	ND	0.068	0.007	
155	0	0.955	0.684	0.606	0.525	ND	0.617	1.042	
156	6	6.027	0.357	0.264	0.046	ND	0.497	0.499	0.5
157	12	11.426	2.093	1.385	0.195	ND	0.833	61.001	-4.8
158	18	17.560	0.882	0.439	0.412	ND	0.712	1.030	-2.4
159	0	5.719	1.844	0.348	0.959	ND	2.112	0.566	
160	0	ND	0.170	0.157	ND	ND	0.062	ND	
161	0	ND	0.115	0.164	ND	ND	0.069	0.002	
162	0	ND	0.150	0.158	ND	ND	0.066	0.074	
163	0	ND	0.140	0.158	ND	ND	0.060	0.067	
164	0	ND	0.120	0.156	ND	ND	0.062	0.091	
165	6	5.624	0.633	0.197	0.025	ND	0.318	3.926	-6.3
166	12	12.339	1.204	0.237	0.081	ND	0.358	7.870	2.8
167	18	18.034	1.540	0.255	0.128	ND	0.825	13.258	0.2
168	0	0.001	0.118	0.159	ND	ND	0.065	0.057	
169	0	ND	0.188	0.160	ND	ND	0.064	0.057	
170	0	ND	0.123	0.156	ND	ND	0.066	0.034	
171	6	0.580	0.251	0.197	0.045	ND	0.335	0.161	-90.3

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
172	12	11.485	0.402	0.224	0.089	ND	0.731	0.144	-4.3
173	0	ND	0.117	0.156	ND	ND	0.066	0.063	
174	12	10.809	0.851	0.210	0.780	ND	0.704	0.082	-9.9
175	18	16.136	0.698	0.232	0.723	ND	1.162	0.164	-10.4
176	0	0.001	0.196	0.160	0.073	ND	0.063	0.035	
177	0	ND	0.108	0.157	ND	ND	0.061	0.033	
178	0	ND	0.121	0.158	ND	ND	0.061	0.039	
179	6	0.578	0.175	0.184	0.032	ND	0.470	0.058	-90.4
180	0	0.001	0.131	0.159	ND	ND	0.069	0.046	
181	6	5.042	0.733	1.205	0.097	ND	0.612	22.745	-16.0
182	12	9.919	1.497	1.061	0.190	ND	0.711	39.548	-17.3
183	18	15.305	2.440	1.505	0.307	ND	1.141	62.429	-15.0
184	0	ND	0.116	0.155	ND	ND	0.061	0.044	
185	0	ND	0.183	0.158	ND	ND	0.060	0.047	
186	6	0.602	0.313	0.267	0.099	ND	0.438	0.058	-90.0
187	0	ND	0.131	0.162	ND	ND	0.068	0.049	
188	18	18.153	1.367	0.315	1.139	ND	1.471	0.210	0.8
189	0	0.001	0.179	0.160	ND	ND	0.062	0.036	
190	6	6.122	0.687	0.181	0.337	ND	0.441	0.200	2.0
191	12	12.221	1.038	0.210	0.671	ND	0.718	0.308	1.8
192	0	ND	0.140	0.162	ND	ND	0.066	0.039	
193	0	ND	0.108	0.155	ND	ND	0.063	0.052	
194	0	ND	0.123	0.166	ND	ND	0.066	0.041	
195	16	14.842	6.784	5.994	1.582	0.092	ND	5.505	-7.2
196	0	13.569	27.610	26.057	11.809	3.010	9.977	1.561	

Sample code	NIC declared (mg/mL)	NIC measured (mg/mL)	MYO (µg/mL)	NNIC (µg/mL)	COT (µg/mL)	NCOT (µg/mL)	ABS (µg/mL)	ATB (µg/mL)	Δ declared (%)
197	18	0.011	ND	0.320	0.320	ND	ND	ND	-99.9
198	0	0.017	ND	ND	0.311	ND	ND	ND	
199	0	0.001	ND	ND	0.308	ND	ND	ND	
200	0	0.001	ND	ND	0.309	ND	ND	ND	
201	48	46.422	62.851	26.653	40.342	0.468	ND	7.396	-3.3
202	18	10.970	18.459	1.745	5.181	0.213	27.977	14.758	-39.1
203	12	10.029	17.231	2.141	5.237	0.137	23.884	18.739	-16.4
204	0	1.617	1.658	0.886	0.537	ND	ND	0.448	
205	0	0.004	ND	ND	0.325	ND	ND	ND	
206	4	4.595	2.177	0.858	1.697	ND	ND	0.363	14.9
207	12	11.868	9.232	6.783	2.051	ND	ND	1.624	-1.1
208	12	9.653	4.066	0.788	1.029	0.718	ND	0.513	-19.6
209	6	5.898	4.126	0.575	3.967	0.010	ND	0.317	-1.7
210	12	10.993	6.082	1.162	1.389	ND	30.552	34.663	-8.4
211	11	10.711	7.266	0.964	6.364	0.091	ND	0.721	-2.6
212	0	0.003	ND	ND	0.316	ND	ND	ND	
213	6	6.133	4.166	5.244	0.776	ND	25.055	38.378	2.2
214	12	11.444	3.317	0.734	3.099	ND	ND	0.850	-4.6
215	8	5.945	1.589	0.570	0.643	ND	ND	1.055	-25.7
216	8	5.841	3.936	0.545	1.318	ND	ND	4.771	-27.0

**Table A- 3: Results for tobacco alkaloids in cigarette tobacco extracts**

<b>Sample code</b>	<b>NIC (mg/mL)</b>	<b>MYO (µg/mL)</b>	<b>NNIC (µg/mL)</b>	<b>COT (µg/mL)</b>	<b>NCOT (µg/mL)</b>	<b>ABS (µg/mL)</b>	<b>ATB (µg/mL)</b>
Cigarette 1	15.759	6.815	484.033	82.742	1.445	64.010	343.027
Cigarette 2	15.878	7.729	488.820	112.834	2.180	86.295	386.597
Cigarette 3	14.096	9.595	584.537	112.081	2.072	66.357	304.754
Cigarette 4 (average of two determinations)	12.211	6.399	391.155	88.880	1.608	60.226	328.569
Cigarette 3R4F (average of two determinations)	15.032	6.307	413.240	94.479	1.912	82.133	344.703

## **ANNEX 2 – Analytical method for the evaluation of flavour profiles**

### **A2.1. Analysis of volatile constituents of e-liquids by thermal desorption high-resolution gas chromatography QTOF mass spectrometry**

#### **1) Standards and reagents**

Isotopically labelled standard 2-ethylphenol-D<sub>10</sub> was purchased from CDN isotopes (Quebec, Canada). Spiking solution of labelled standard of about 30 µg/mL was prepared gravimetrically in methanol. Methanol of LC-MS grade was obtained from VWR (Leuven, Belgium).

#### **2) Sample preparation**

For the analysis of volatile compounds, 30 µL of homogenized sample was taken to the vial and dissolved in 900 µL methanol. Labelled internal standard 2-ethylphenol-D<sub>10</sub> was added (100 µL, 30 µg/mL), the vial was vigorously shaken and placed on the GC/Q-TOF-MS autosampler for the analysis.

#### **3) Thermal desorption GC-QTOF analysis**

##### Operating conditions

Six µL of the sample was injected by MPS sampler into the liner of the thermal desorption unit (TDU) containing a micro-vial insert. The TDU was programmed from 20 °C held for 0.1 min to 100 °C at 30 °C/min held for 15 min with purge flow 100 mL/min. Volatile compounds were trapped in the PTV inlet on Tenax TA packed liner at 15 °C. Trapped compounds were injected onto the HP-5MS GC column (30 m x 250 µm x 0.25 µm, Agilent Technologies) with split ratio 15:1 while programming the PTV inlet from 15 °C held for 0.1 min to 270 °C at 12 °C/s held for 20 min. The GC oven was programmed from 45 °C (held for 2 min) to 210 °C at 4°C/min further to 300 °C at 10 °C/min (held for 10 min). Helium was used as a carrier gas at 1 mL/min flow rate. The transfer line temperature was set at 300 °C. The Q-TOF-MS was operated at a mass range of m/z 45-450. The data acquisition rate was 5 Hz in 2 GHz extended dynamic range (EDR) mode.

##### Instrument configuration

The TDU-GC/Q-TOF-MS system consisted of: A gas chromatograph (GC) 7890A (Agilent Technologies, Santa Clara, CA, USA) was equipped with a cooled injection system CIS, a programmable temperature vaporizing (PTV) inlet (Gerstel, Mülheim an der Ruhr, Germany) and TDU thermal desorption unit (Gerstel) operating automatically in conjunction with MurtiPurpose Sampler MPS (Gerstel). The GC was coupled to an Agilent 7200 Accurate Mass Q-TOF MS (Agilent Technologies). Operation of the instrument was

controlled by MassHunter GC/MS Acquisition version B.07.03.2129 (Agilent Technologies) and Gerstel Maestro 1 version 1.4.31.10/3.5 (Gerstel).

#### **4) Data Analysis**

Mass Hunter Qualitative Analysis version B.07.00 (Agilent Technologies), Mass Hunter Unknowns Analysis version B.07.01 (Agilent Technologies), Mass Hunter Quantitative Analysis for QTOF version B.07.01 (Agilent Technologies), Mass Profiler Professional version 12.5 (Agilent Technologies), XCMS<sup>TM</sup> version v3.5.1 (Scripps Research Institute, La Jolla, CA, USA) and Mass Mountaineer<sup>TM</sup> version 2.00.3.1 (RBC software, Reabody, MA, USA) were used for the data analysis. Multivariate statistics were applied using SIMCA version 14.1.0.2047 (MKS Umetrics, Malmo, Sweden) and Statgraphics, version 15.2.06 (StatPoint Technologies, Warrenton, VA, USA).

#### **5) Analytical precision**

Repeatability expressed as a relative standard deviation (RSD) of the absolute responses of labelled standard spiked in 12 different e-liquids was 2%. Intermediate precision calculated as RSD of labelled standard response analysed in the period of two months was 13%.

## A2.2. Test samples

In total 108 samples of e-liquids comprising different producers and flavour characteristics (tobacco, mint, vanilla, fruits and others) were selected for the volatile analysis (Table 1).

**Table A- 4: Samples analysed for profile of volatiles**

Sample code	Description	Produced in	NIC declared (mg/mL)	Flavour
F04a	Caramel Machiato	Netherlands	0	Coffee
F04b	Caramel Machiato	Netherlands	12	Coffee
F04c	Caramel Machiato	Netherlands	18	Coffee
F05	Cappuccino	Netherlands	0	Coffee
F06a	Coffee	Spain	0	Coffee
F08	Cappuccino	Croatia	18	Coffee
F09	Coffee	China	6	Coffee
D01	Passion Fruit	Netherlands	0	Fruit
D02	Mandarin	Netherlands	0	Fruit
D03a	Strawberry	Netherlands	0	Fruit
D03b	Strawberry	Netherlands	6	Fruit
D03c	Strawberry	Netherlands	12	Fruit
D03d	Strawberry	Netherlands	18	Fruit
D04	Coconut	Netherlands	0	Fruit
D05	Pear	Netherlands	0	Fruit
D06	Strawberry	China	18	Fruit
D07a	Erdbeer fresh	Germany	0	Fruit
D07b	Erdbeer fresh	Germany	20	Fruit
D08a	Erdbeer	Germany	10	Fruit
D08a	Erdbeer	Germany	20	Fruit
E11	Honigmelone	Germany	10	Fruit
E12	Himbeer	Germany	10	Fruit
E13	Frucht-mix Tropic	Germany	10	Fruit
E14	Amarena Kirsch	Germany	10	Fruit
K01	Apple	China	18	Fruit
K02	Cherry	China	24	Fruit
K03	Apfel-kuchen	Germany	0	Fruit
K04	Kirsch Amarena fresh	Germany	0	Fruit
K05	Zitrone fresh	Germany	0	Fruit
K06	Apfel fresh	Germany	0	Fruit
K07	Brombeer fresh	Germany	0	Fruit
K08	Pfirsich Maracuja fresh	Germany	0	Fruit
K09	Wassermelone fresh	Germany	0	Fruit
K10	Himbeer fresh	Germany	0	Fruit
K11	Walderdbeer fresh	Germany	0	Fruit

Sample code	Description	Produced in	NIC declared (mg/mL)	Flavour
K12	Cherry Limeade	USA	0	Fruit
B02	Menthol	Netherlands	0	Mint
B03	Arctic Mint	Netherlands	0	Mint
B04a	Ice Mint	Netherlands	0	Mint
B04b	Ice Mint	Netherlands	6	Mint
B04c	Ice Mint	Netherlands	12	Mint
B04d	Ice Mint	Netherlands	18	Mint
B05	Mint	Netherlands	0	Mint
B06	Menthol	China	0	Mint
E01a	Banana Split	Netherlands	0	Other
E02	Cotton Candy	Netherlands	0	Other
E03	White Chocolate	Netherlands	0	Other
E04	Banana Chocolate	Netherlands	0	Other
E05	Pumpkin Pie	Netherlands	0	Other
E06	Biscuit	Italy	0	Other
E07	Toffee	Germany	0	Other
E08	Marzipan	Germany	0	Other
E09	Honig	Germany	0	Other
E10	Cookie	Germany	0	Other
F07	Karamell Sahne	Germany	0	Other
A01	Arabic tobacco	Netherlands	0	Tobacco
A02	USA Mix	Netherlands	0	Tobacco
A03	Turkish blend	Netherlands	0	Tobacco
A04	Southeast County	Netherlands	0	Tobacco
A05	Cuba Cigar	Netherlands	0	Tobacco
A06a	Coco & Havana	Netherlands	0	Tobacco
A06b	Coco & Havana	Netherlands	6	Tobacco
A06c	Coco & Havana	Netherlands	2	Tobacco
A06d	Coco & Havana	Netherlands	18	Tobacco
A07	Honey Toasted Tobacco	Netherlands	0	Tobacco
A09	Old Dry Tobacco	Italy	0	Tobacco
A10	The Palm	Netherlands	0	Tobacco
A11	Tobacco Natural	Croatia	48	Tobacco
A12	Desert ship	France	12	Tobacco
A13	Marbi	EU	no Info	Tobacco
A14	Real tobacco	EU	no Info	Tobacco
A15	Traditional tobacco	Czech Republic	12	Tobacco
A16	Tobacco	China	0	Tobacco
A17	Virginia Tobacco	China	18	Tobacco
A18	Tobacco	China	18	Tobacco
A19a	Calamity Jane Tobacco	USA	0	Tobacco



Sample code	Description	Produced in	NIC declared (mg/mL)	Flavour
A19b	Calamity Jane Tobacco	USA	12	Tobacco
A19c	Calamity Jane Tobacco	USA	18	Tobacco
A20a	Ol' River Tobacco	USA	0	Tobacco
A20b	Ol' River Tobacco	USA	18	Tobacco
A21	Rocket Blend	USA	0	Tobacco
A22	Tobacco	Germany	0	Tobacco
A23	Black Mamba	Germany	0	Tobacco
A24	Black Fire	Germany	0	Tobacco
A25	Pfeife	Germany	0	Tobacco
A26	Kentucky	Germany	0	Tobacco
A27	American Blend	Germany	0	Tobacco
A30	Tabac Fresh	France	0	Tobacco
A31	Tabac Regular	France	0	Tobacco
A32	Tabac USA	France	0	Tobacco
A33	Tabac British	France	0	Tobacco
A34	Strong Blondy	France	0	Tobacco
A35	Goldy	France	0	Tobacco
A36	Le Gitan	France	0	Tobacco
A37	Tabac Des Iles	France	0	Tobacco
B01	Tobacco Mint	Netherlands	0	Tobacco
B07	Tabac Mint	France	0	Tobacco
C02	Shisha Vanille	Netherlands	0	Tobacco
E15	Tabac Caramel	France	0	Tobacco
F03	Shisha Coffee	Netherlands	0	Tobacco
F10	Tabac Café	France	0	Tobacco
C01	Vanilla	Netherlands	0	Vanilla
C03a	Vanilla Orange	Netherlands	0	Vanilla
C03b	Vanilla Orange	Netherlands	6	Vanilla
C03c	Vanilla Orange	Netherlands	12	Vanilla
C03d	Vanilla Orange	Netherlands	18	Vanilla
C04	Vanille	France	0	Vanilla
C05	Ice Vanille	Netherlands	0	Vanilla

### A2.3. Compound identification

Approximately 100 components were found in each e-liquid sample with Mass Hunter search using high resolution. Identification of the compounds was performed with the NIST library mass spectra search and based on compounds linear retention indexes (LRI). In total 90 compounds were tentatively identified in the tobacco flavoured e-liquids (table 2). Majority of these compounds (more than 70) was also reported in the tobacco either as a naturally presented or added as an ingredient for flavour formulation (Rodgman & Perfetti, 2013).

**Table A- 5 Compounds identified in e-liquid products declared as a tobacco flavoured**

(RT: retention time, CAS No: Chemical Abstracts Service number, LRI: linear retention index, LRI (lit): linear retention index retrieved from literature)

No.	RT [min]	Compound	CAS No.	LRI	LRI (lit)*
1	4.149	Methylbutyrate	623-42-7	721	723
2	5.086	methyl 2-methylbutyrate	868-57-5	779	780
3	5.539	Ethylbutyrate	105-54-4	805	805
4	5.679	dihydro-2-methyl-3(2H)-furanone	3188-00-9	810	810
5	5.850	ethyl lactate	97-64-3	816	813
6	6.064	2-methylpyrazine	109-08-0	824	825
7	6.864	2-furanmethanol	98-00-0	855	862
8	7.881	o-xylene	95-47-6	895	891
9	8.138	3-methoxy-1,2-propanediol	623-39-2	903	900
10	8.443	2,5-dimethylpyrazine	123-32-0	912	911
11	8.643	2,3-dimethylpyrazine	5910-89-4	918	919
12	9.101	2,4-dimethyl-1,3-dioxolane-2-methanol	53951-43-2	932	-
13	9.329	2,2-dimethyl-1,3-dioxolane-4-methanol	100-79-8	939	1016
14	9.400	2-butyl-4-methyl-1,3-dioxolane	74094-60-3	941	939
15	9.678	Ethyl-3-oxobutyrate	141-97-9	949	943
16	10.000	Benzaldehyde	100-52-7	960	961
17	10.125	5-methylfurfural	620-02-0	963	962
18	11.393	Trimethylpyrazine	14667-55-1	1001	1000
19	12.064	Acetylpyrazine	22047-25-2	1020	1023
20	12.196	1,1-oxybis-2-propanol	110-98-5	1024	1014
21	12.290	2-hydroxy-3-methyl-2-cyclopentene-1-one	80-71-7	1027	1034
22	12.562	Benzylalcohol	100-51-6	1034	1032
23	13.005	1-methyl-2-pyrrolidine	872-50-4	1047	1045

No.	RT [min]	Compound	CAS No.	LRI	LRI (lit)*
24	13.243	$\gamma$ -caprolactone	695-06-7	1054	1056
25	13.493	2-acetylpyrrole	1072-83-9	1061	1060
26	13.813	3-methyldecane	13151-34-3	1070	1069
27	13.914	cis-linalool oxide	5989-33-3	1073	1072
28	14.255	allyl hexanoate	123-68-2	1082	1080
29	14.396	Tetramethylpyrazine	1124-11-4	1085	1085
30	14.474	trans-linalool oxide	34995-77-2	1089	1089
31	14.656	1-monoacetin	106-61-6	1093	1091
32	14.868	Undecane	1120-21-4	1100	1100
33	15.047	Nonanal	124-19-6	1105	1104
34	15.341	Maltol	118-71-8	1113	1108
35	15.380	2-phenylethanol	60-12-8	1114	1116
36	15.634	3-ethyl-2-hydroxy-2-cyclopenten-1-one	21835-01-8	1121	1100
37	16.443	2,6,6,-trimethyl-2-cyclohexene-1,4-dione	1125-21-9	1143	1140
38	16.577	2-hydroxy-3,5,5-trimethyl-cyclohex-2-enone	4883-60-7	1147	1150
39	17.403	Ethylbenzoate	93-89-0	1170	1170
40	17.484	Menthol	15356-70-4	1173	1171
41	17.814	diethylester succinic acid	123-25-1	1182	1181
42	17.906	$\alpha$ -monopropionin	624-47-5	1185	1190
43	18.073	2-(2-butoxyethoxy)-ethanol	112-34-5	1190	1192
44	18.357	Ethylmaltol	4940-11-8	1197	1197
45	19.122	2-phenoxyethanol	122-99-6	1220	1221
46	19.240	1,2-benzisothiazole	272-16-2	1223	1221
47	19.422	3,7-dimethyl-6-octen-1-ol	106-22-9	1228	1228
48	20.005	Ethylphenylacetate	101-97-3	1245	1244
49	20.305	4-methoxybenzaldehyde	123-11-5	1254	1251
50	20.449	$\gamma$ -octalactone	104-50-7	1259	1262
51	20.760	4-methyl-2-phenyl-1,3-dioxolane	2568-25-4	1268	1272
52	20.847	trans-cinnamaldehyde	14371-10-9	1270	1266
53	21.015	Perillaldehyde	2111-75-3	1275	1272
54	21.268	p-anisyl alcohol	105-13-5	1283	1279
55	21.381	1-methoxy-4-(1-propenyl)-benzene	104-46-1	1285	1289

No.	RT [min]	Compound	CAS No.	LRI	LRI (lit)*
56	21.921	Carvacrol	499-75-2	1301	1298
57	22.962	Piperonal	120-57-0	1331	1329
58	23.430	Nicotine	54-11-5	1348	1341
59	23.583	1-hydroxy-2,4,4-trimethylpentan-3-yl 2-methylpropanoate	74367-33-2	1352	1351
60	23.602	4-acetylanisole	100-06-1	1353	1345
61	23.634	Triacetin	102-76-1	1354	1356
62	23.764	p-eugenol	97-53-0	1358	1356
63	23.936	$\gamma$ -nonalactone	104-61-0	1363	1366
64	24.280	3-hydroxy-2,4,4-trimethylpentyl 2-methylpropanoate	74367-34-3	1374	1376
65	24.661	trans- $\beta$ -damascenone	23726-93-4	1386	1391
66	24.942	trans- $\alpha$ -damascone	24720-09-0	1394	1395
67	24.987	2-phenylethylisobutyrate	103-48-0	1396	1396
68	25.075	Vanillin	121-33-5	1399	1391
69	25.287	Methyleugenol	93-15-2	1408	1405
70	25.609	1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-buten-1-one	35044-68-9	1415	1412
71	25.771	p-anisyl acetate	104-21-2	1420	1419
72	25.823	Myosmine	532-12-7	1421	1427
73	26.257	Coumarin	91-64-5	1437	1439
74	26.771	trans-geranylacetone	3796-70-1	1451	1453
75	26.875	ethyl vanillin	121-32-4	1454	1452
76	27.238	$\gamma$ -decalactone	706-14-9	1469	1472
77	27.654	Anabasine	494-52-0	1483	1486
78	27.762	Nicotyrine	487-19-4	1486	1488
79	27.822	$\beta$ -ionone	79-77-6	1487	1485
80	28.564	2,4-ditertbutylphenol	96-76-4	1512	1513
81	28.577	Anatabine	2743-90-0	1512	1510
82	29.144	2,3-dipyridyl	581-50-0	1532	1536
83	29.904	6-methylcoumarin	92-48-8	1558	1549
84	31.021	Diethylphthalate	84-66-2	1596	1594
85	35.647	Benzylbenzoate	120-51-4	1765	1761

No.	RT [min]	Compound	CAS No.	LRI	LRI (lit)*
86	37.565	Neophytadiene	504-961	1839	1840
87	38.573	1-hexadecanol	36653-82-4	1879	1880
88	40.570	n-hexadecanoic acid	57-10-3	1961	1963
89	43.430	1-octadecanol	112-92-5	2083	2084
90	43.670	Benzylcinnamate	103-41-3	2094	2096

\* LRI were searched in databases such as <http://www.flavornet.org/>,  
<http://www.pherobase.com/>,  
<http://webbook.nist.gov/chemistry/>,  
<https://pubchem.ncbi.nlm.nih.gov/>.

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## List of figures

Figure 1: Difference between the NIC concentrations declared on the label and the measured concentration in the analysed samples. ....	9
Figure 2: Relative concentrations of minor alkaloids in traditional cigarettes. ....	11
Figure 3: Relative concentrations of minor alkaloids in e-liquids. ....	11
Figure 4: Principle component analysis – Score plot of PC1 and PC2 for nicotine containing e-liquids and cigarette tobacco extracts .....	12
Figure 5: Prevalence of measured substances in different e-liquid categories .....	15
Figure 6: PCA score plot of e-liquid volatiles: .....	15



## List of tables

Table 1: Chemical structures and acronyms of the analysed substances.....	8
Table 2: Number of test samples per flavour category .....	14
Table 3: Cross-validation of the PLS-DA model based on five flavour groups.....	16
Table 4: Individual results from validation of the PLS-DA model based on five flavour groups with independent samples.....	17
Table 5: Cross-validation of the PLS-DA model based on two flavour groups.....	17
Table 6: Individual results from validation of the PLS-DA model based on two flavour groups with independent samples.....	18
Table 7: Accuracy table for cross validation of decision tree model .....	18
Table 8: Validation of decision tree model with independent samples.....	19



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