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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

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ABSTRACT

The electronic cigarette (e-cigarette) has emerged as a popular alternative to the traditional hazardous tobacco cigarette. The substantial increase in e-cigarette use also urgently calls for controlling the quality of e-cigarette refill liquid products (e-liquids). Currently, the most important quality indicator of e-liquid products is the quantification of nicotine and its related impurities. Although different methods have been published to measure nicotine and impurity levels, 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. Therefore, in this study, a simple dilute-and-shoot UHPLC-DAD method has been developed and validated for the simultaneous quantification of nicotine and its alkaloid impurities in electronic cigarette refills. An optimal separation of the alkaloids was achieved in a runtime of 11 min. The method was successfully validated using the "total error" approach in accordance with the validation requirements of ISO-17025. During this validation, interference between the target components and a number of popular flavouring compounds such as vanillin, maltol, ethylacetate, etc. could be excluded. In addition, small changes to the column temperature, pH and molar concentration of the mobile phase buffer were deliberately introduced in order to assess the robustness of the method. Only a slightly different outcome between the newly developed UV-detection method and the targeted MS approach was found, due to the sensitivity of the different detection techniques. However, in the context of quality control of nicotine related impurities, for which the European Pharmacopoeia limits are currently applied, the sensitivity of the UHPLC-DAD method was found to be within the acceptable range. Despite the somewhat lower selectivity of the newly developed UV-detection technique *versus* a targeted LC-MS/MS approach, it may be concluded that this method is a suitable alternative for quality control purposes.

1. Introduction

As shown by the increasing sales of the past years, the e-cigarette has become widely popular [1]. The e-cigarette market is characterized 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 [2]. 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 [3].

The requirements to e-cigarettes on the European market are regulated by the Tobacco Product Directive (TPD)[4]. 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

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the ingredients. These gaps in the Directive could have an impact on the quality and therefore the safety of the e-liquids [5]. Quality standards for e-liquids are thus highly needed, more so because of the recent discovery of counterfeit 'e-liquids' [6]. 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 [7].

One of the most investigated ingredients in e-liquids is nicotine. The amount of nicotine and compliance with the labeled 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 labeling practices [8–10]. Yet, low quality e-liquids are not only characterized by nicotine mismatch or mislabeling due to poor manufacturing conditions, but also by the presence of impurities.

One of these impurities are the nicotine related impurities that consist of nornicotine, anabasine, anatabine, myosmine, cotinine, β-nicotyrine and nicotine-N-oxide. 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 [11]. 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 [12]. Hence, e-liquids may contain these alkaloid impurities when tobacco extracts have been used to obtain a tobacco flavour [13]. 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 [10,13–19]. The quantification of nicotine is easily done by UV detection because of its chromophore. Furthermore, analysis with GC is feasible due to its volatility [20]. 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 [21]. 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 [22]. 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 [18,19,21,23–25]. 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 Supplementary Table S1 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 Internation Conference of Harmonisation (ICH) guidelines using accuracy profiles [10]. 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 and 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, methylaminobenzoaat and methylmyristate. The solvents acetonitrile, methanol, acetone and ethylactetate 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 1.

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/water 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 quantification analysis. The validation samples were prepared in triplicate at three concentration levels (Table 1). During 5 days, samples were prepared at 3 spiking levels each with 3 replicates. Thus, in total 9 samples were prepared every day similar to the calibration curve for the quantification 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

Table 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
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

Table 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, 1.7 μm, 100 mm, 2.1 mm) with Van Guard BEH C18 pre-kolom (1.7 μm, 2.1 mm, 5 mm)	Acquity UPLC BEH C18 (Waters, 1.7 μm, 100 mm, 2.1 mm)
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		
Flow	0.4 ml/min	0.5 ml/min
Injection volume	10μl	10 μl
Detection	254 nm, 234 nm en 285 nm	MRM transitions (Table 3)
Column temperature	30 °C	45 °C

'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 at the same retention time as 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 2. Acetonitrile was used as the strong needle wash solvent and 95% water and 5% acetonitrile as the weak needle wash solvent.

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 myosine 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 2. The analytes were measured in positive electrospray ionization (ESI) mode. The monitored MRM transitions (2 for each analyte) and the compound specific parameters can be found in Table 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.

Table 3
Monitored MRM transitions of the LC-MS/MS method for the analysis of nicotine and its related impurities with their specific MS-parameters.

	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

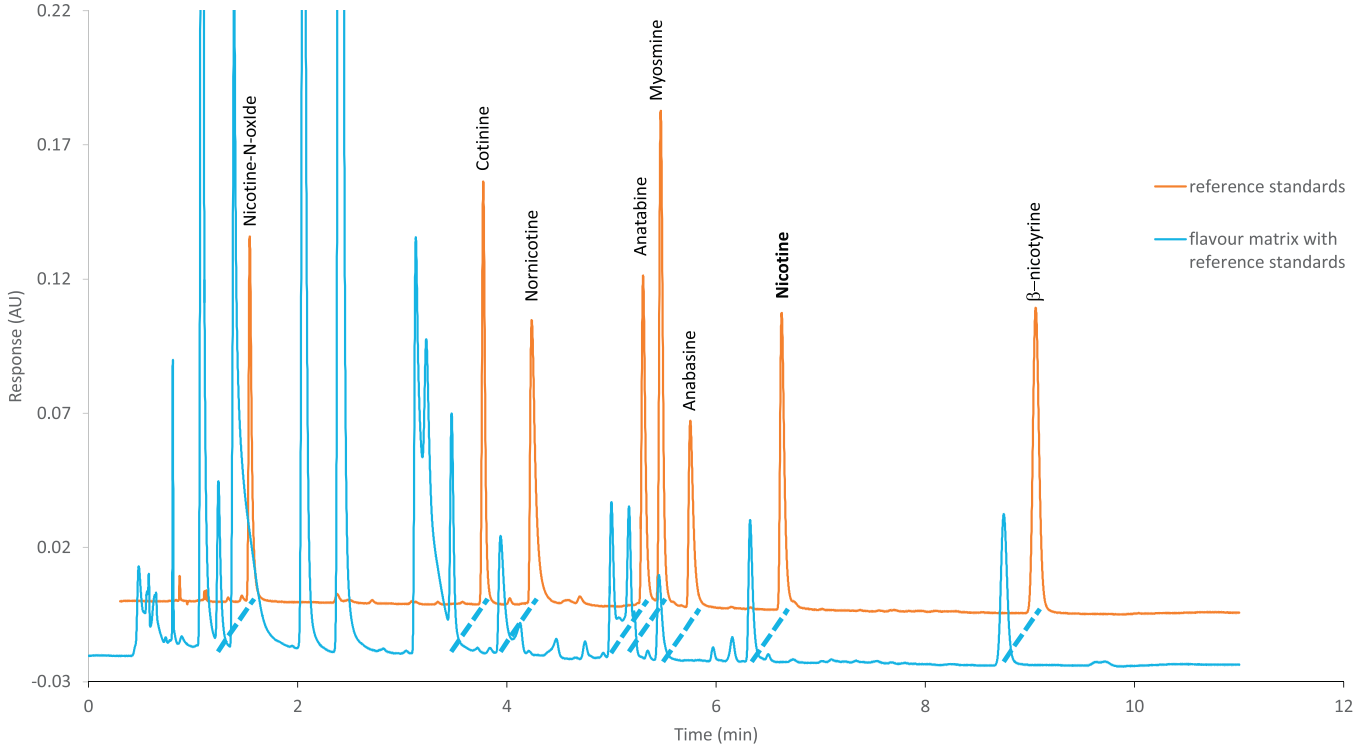

Fig. 1. Chromatogram of the target components obtained with the optimized UPLC-DAD method. Nicotine-N-oxide (1.26 min), cotinine (3.51 min), nornicotine (3.91 min), anatabine (4.99 min), myosmine (5.16 min), anabasine (5.39 min), nicotine (6.44 min), β-nicotyrine (8.98 min).

The screening method was validated for the intended purpose according to the validation guidelines [26,27]. 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 × 1,5 IP). A target component was identified correctly when the following criteria were met: 1) max 5 % deviation from the retention time; the ratio of the 2 fragment ions differed not more than 30%; the S/N ratio was larger than 10. In Fig. 2 a representative chromatogram is shown of an analysis of the nicotine impurities reference standards with the targeted screening method.

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; 5 samples were bought in Belgian vape shops and 5 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

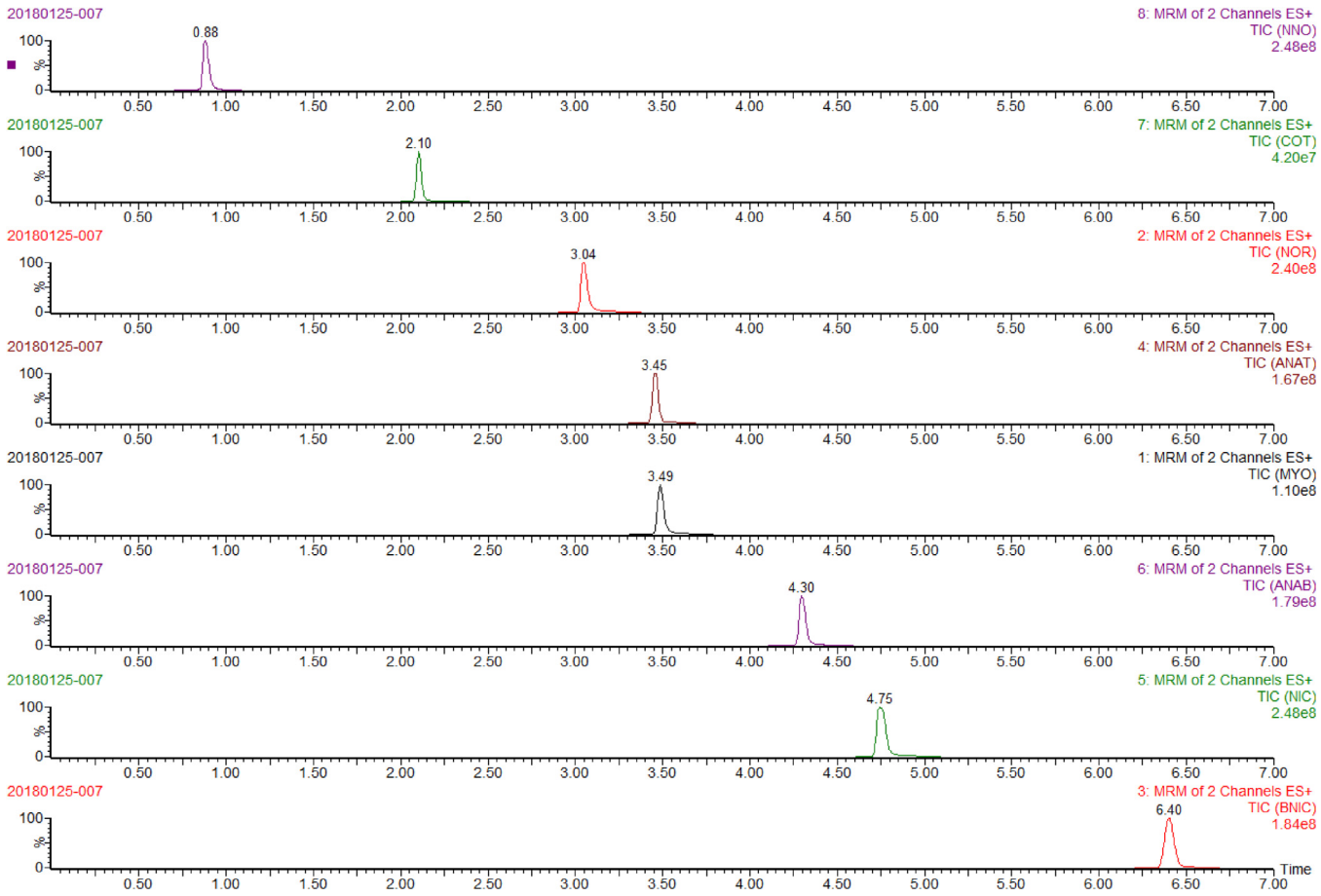

Fig. 2. Representative LC-MS/MS chromatogram of the target components with as response the sum of both selected MRM transitions.

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 [28]. 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. The UV-spectrum for each component is given in Supplemental Figure S1.

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 (Fig. 1). This does not imply that there are no interferences of the target components with other flavours/additives. There are more than hundreds

Table 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

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 [4], 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 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 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 [29].

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 5).

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 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 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.

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 Fig. 3 and the β-expectation tolerance intervals are given in Table 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 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 samples, as recommended by the International Council for Harmonisation (ICH) guidelines [30]. 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 4.

Table 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.

	R^2	Relative bias (%)			Repeatability (%RSD)			Intermediate reproducibility (%RSD)			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]

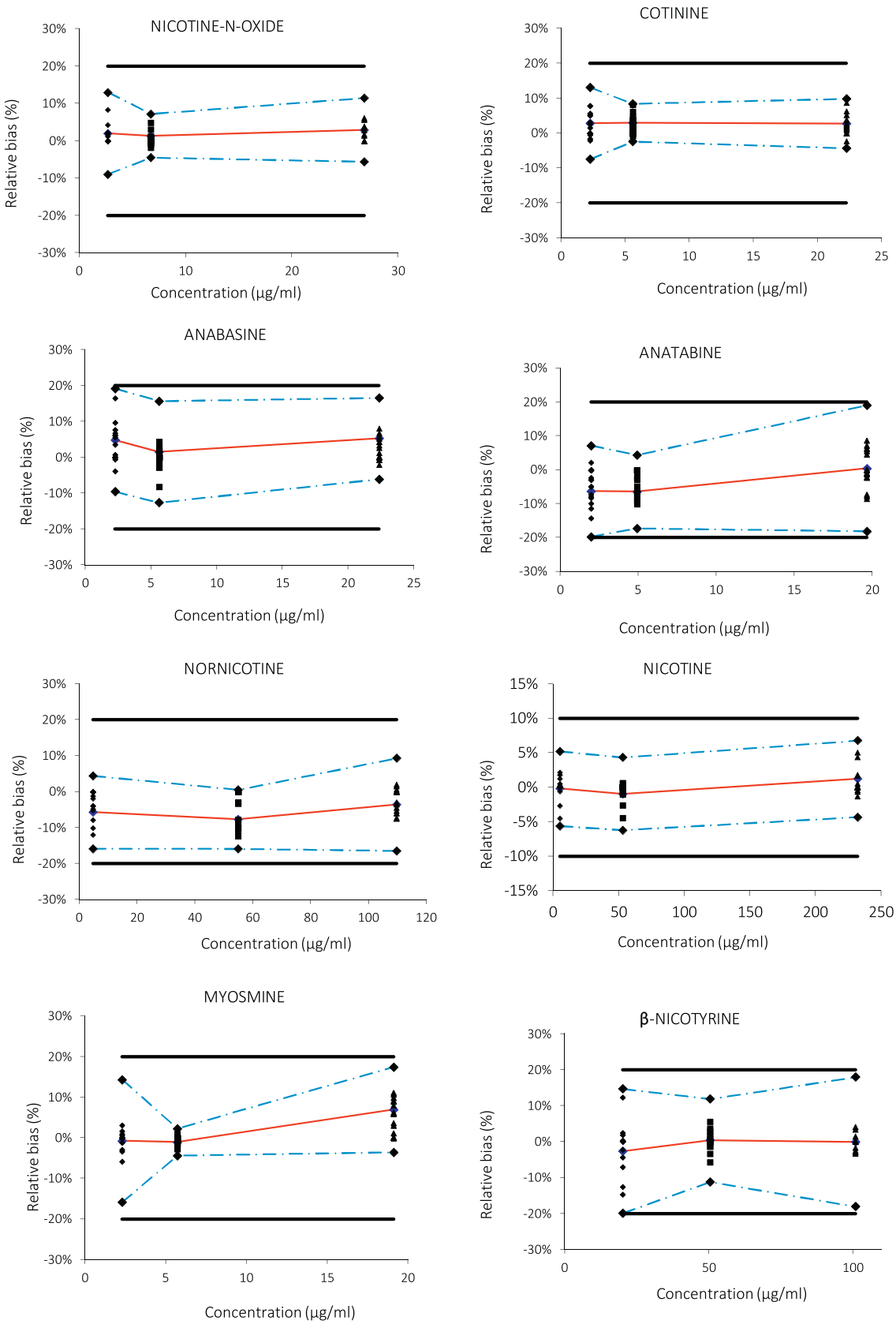

Fig. 3. Accuracy profiles obtained for the target components with β set at 95%. Legend: Relative bias (red solid line), upper and lower β-expectation tolerance limits (dotted blue line), upper and lower acceptance limits set at 10% or 20% (black solid line), relative back-calculated concentrations per spiking level(♦▲ ■). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 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.

	µg/ml	Matrix 1 average±SD	Matrix 2 average±SD	Matrix 3 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	102.0 2± 2.34

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.

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. 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

3.2.8. Robustness

The robustness of the UHPLC-DAD method was investigated. As can be seen in Fig. 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

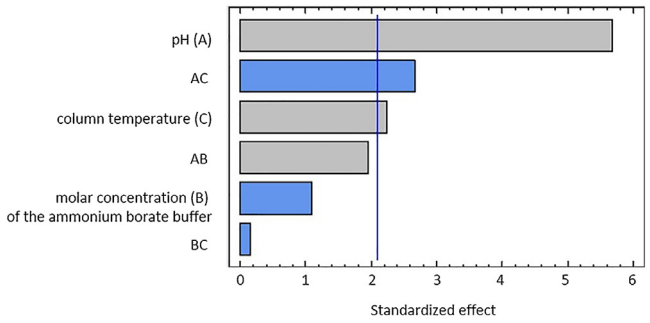

Fig. 4. Pareto chart of the effects for the robustness study. The effect is represented by the resolution of the critical peak pair. The investigated factors were the pH (A) and molar concentration (B) of the ammonium borate buffer and the column temperature (C).

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.

3.3. Proof of applicability

To proof the applicability of the UHPLC-DAD method, a small sample set of e-liquids on the market 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 7. Out of the 10 samples investigated, 4 samples were labelled as zero liquids (INT 1, INT2) 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

Table 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
	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.

of nicotine were present in the other 2 (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.

Nicotine-containing e-liquid samples were analysed as well. In general, all nicotine-containing samples contained the related-nicotine impurities (Table 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 [10]. 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.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.03.002.

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