Development and validation of an ultrahigh-performance liquid chromatography method for identity, assay and impurity testing of nicotine and preservatives in nicotine nasal spray

Amane Kandas

Degree Project in Analytical Chemistry, 2021
Department of Chemistry
Lund University
Sweden

BSc / MSc, 30 hp

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Examiner:
Peter Spégel
Centre for Analysis and Synthesis
Dept. of Chemistry
Lund University

Main supervisor:
Margareta Sandahl
Centre for Analysis and Synthesis
Dept. of Chemistry
Lund University

Supervisor:
Tobias Juhlin
Analytical Chemist
QC Finished Products
McNeil AB

Lund University

Department of Chemistry

Centre for Analysis and Synthesis

P.O. Box 124

SE-221 00 Lund, Sweden

Analysis of nicotine nasal spray – method development and validation

Nicotine nasal spray is produced at McNeil AB, with the aim to help people stop smoking, and thereby decrease the deaths caused by smoking. Since nicotine nasal spray is considered as a pharmaceutical product, the analysis of the product needs to fulfil regulatory requirements. Currently used method was issued in the 20th century, which due to an update in the regulatory requirements, also need to be updated. When developing an analysis method of a pharmaceutical product, a validation of the new method is essential to investigate if the requirements are fulfilled.

The method used today was developed year 2019, however this method needed further development before implementation. The aim of this project is to develop this last-mentioned analysis method of nicotine nasal spray, and thereafter to perform a validation by examining various parameters. The analysis method was performed by using ultrahigh-performance liquid chromatography, a commonly used analytical technique. Analytes that were studied were nicotine, which is the active pharmaceutical ingredient, preservatives, and degradants from nicotine. The goal was to find the chromatographic conditions that lead to the best resolution, and to obtain a method that fulfil the regulatory requirements. Also, the new method was aimed to be greener than currently used method.

Overall, this project has been successful where all research questions have been answered. The parameters that were investigated were temperature, flow, pH and gradient of the mobile phase. By using the identified conditions, the new method became greener with a decreased consumption of solvent with more than 40 times. Parameters that were investigated during the validation were: linearity, quantification limit, accuracy, carryover, stability, robustness, precision, specificity, and method equivalence. Also, column equivalence and filter study were investigated. Further investigation should be done regarding the stability and column equivalence, whereas other parameters showed acceptable results. Some advantages of the new method compared to the present method, beyond the less consumed solvent, is the improved accuracy, repeatability, and efficiency, as well as the decreased complexity. Finally, by using the same set-up as other analytical methods at the company, the new method is more practical.

Abstract

Introduction: The topic of this study is to develop and validate an analysis method of nicotine nasal spray produced at McNeil AB.

Background: When developing the analysis method, several parameters were determined in advance to make the method more similar to other analysis methods at the company, making it more practical. However, other parameters were adjusted with the aim to improve the resolution and to fulfil the updated cGMP requirements, where category I, II and IV according to USP <1225> were of interest.

Aims: The research questions to be answered during this project are; (1) what conditions that leads to the highest resolution, (2) Does these conditions fulfil the cGMP requirements, and (3) if the new method is greener than the currently used method.

Methods: The aims have been fulfilled by initially varying the temperature, flow, pH and mobile phase gradient by using UHPLC, followed by a full validation where various performance characteristics were studied. Different sequences for the performance characteristics were used for calculations and comparison with the requirements that needed to be fulfilled.

Results: The developed method improved the resolution between the analytes as well as the complexity was decreased. All regulatory requirements were achieved except for the robustness. However, the sample solution was only stable for 45 hours, where the aim was to have a longer stability. Thereby, further investigation should be done on the stability. Also, column equivalence did not fulfil the requirement. The new analysis method was significantly greener, with more than 40 times less consumed solvent.

Conclusion: The developed method showed good results and will be a good implementation for the company, yet further investigations should be done before sending the validation report for approval.

Keywords: Nicotine – Nicotine nasal spray – Ultrahigh-performance liquid chromatography – Development – Validation

Acknowledgement

To start with, I would like to thank McNeil AB for making it possible for me to perform this degree project at their company. The task has been challenging, but most important, the project has taught me how to attack such framing of questions. It has been rewarding to work with a validation for future aspects. The assistance provided by Tobias Juhlin during the entire project is highly appreciated. All the way from start, where the validation was planned, to the end of the project, where the validation was implemented and examined. Having friendly chats as well as academic discussions was of high value and have made this project a lot funnier.

I would also like to thank my examiner, Peter Spégel, and my supervisor, Margareta Sandahl, for giving me feedback and for providing guidance throughout my project. Furthermore, to Charlotta Turner for helping me with green chemistry questions as well as giving me feedback on my work. I want to send many thanks to Zeinab Hojeij who helped me with the investigation of the intermediate precision and for being supportive and kind during my project. I would also like to thank Waters Sverige AB, who offered the premier column, which was of high interest to test on the new method. Finally, my biggest thanks to my family and my husband Jakub for being understanding and believing in me during my education and all hours I have spent on this project.

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1 List of abbreviations

AcN	Acetonitrile
API	Active pharmaceutical ingredient
BEH	Ethylene bridge hybrid
cGMP	Current good manufacture procedure
DL	Detection limit
DOE	Design of experiments
EDTA	Ethylenediamine tetra-acetic acid
HPLC	High-performance liquid chromatography
ICH	International council on harmonization
LC	Label claim
MeOH	Methanol
MNP	Methyl-3-nicotinoylpyrrolidine
MS	Mass spectrometry
N/A	Not applicable
NF	National formulary
NHT	(-)-Nicotine hydrogen tartrate dihydrate
NIC	Nicotine
Nmt	Not more than
NNS	Nicotine nasal spray
NRS	Nicotine related substances
PDA	Photodiode array
PON	Pseudo-oxy-nicotine
QL	Quantitation limit
RF	Response factor
RSD	Relative standard deviation
SL	Specification limit
SST	System suitability test
TLC	Thin-layer chromatography
UHPLC	Ultrahigh-performance liquid chromatography
USP	United States pharmacopeia

2 Introduction

2.1 Background

One of the world's biggest health threats is tobacco, used by 1.3 billion people, whereas more than eight million die due to diseases as a cause of tobacco each year. The most common form of tobacco use is smoking, where the addiction is due to the chemical substance nicotine, shown in Figure 1. [1]

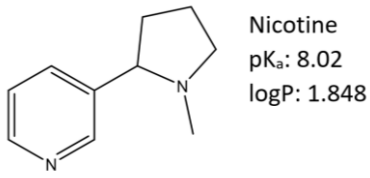

Figure 1: Molecular structure of nicotine.

In average, 1-1.5 mg of nicotine is absorbed systemically when smoking, where the dominant type is the S-isomer. [2] Nicotine is a weak base with a $pK_{a,1}$ of 3.12 and a $pK_{a,2}$ of 8.02, thus it can be both monoprotonated and diprotonated depending on the pH conditions. [3] The ionized species of nicotine does not cross membranes rapidly, thereby a pH of 6.5 or higher of smoke from cigarettes. When having such pH, nicotine can be considered as unionized and will therefore be well absorbed already through the mouth. The absorption increases rapidly when reaching the small airways and the alveoli of the lungs due to the large surface area. Also, the transfer across the membranes is promoted by the dissolved nicotine in the human lungs, which depends on the pH value of 7.4. When smoking, the nicotine reaches the brain only 10-20 s after the puff of the cigarette, leading to smoking being the most reinforcing and dependence-producing form of nicotine administration. [2]

Common diseases caused by smoking are cancer, stroke, diabetes, and chronic obstructive pulmonary disease. [4] The reason leading to the diseases is the high number of constituents of a tobacco smoke, more than 4000, where different cancer forms mainly develop due to carcinogens. [5] Decreasing the deaths caused by smoking is obtained by reducing the addiction among people, which is the aim of McNeil AB by mainly producing and developing NICORETTE®, the leading trademark in the market within smoking and tobacco cessation. McNeil AB with a focus on smoking and tobacco cessation since year 1970 is located in

Helsingborg, with nicotine gum as a leading product sold worldwide. However, also mouth spray, lozenge, inhaler, and nasal spray are manufactured at the company. [6] The benefit of all NICORETTE® products is the controlled amount of chemicals. The product of interest in this study is Nicotine nasal spray (NNS), which as all other NICORETTE products, reduces cravings for nicotine.

One spray volume of NNS is approximately 50 µl and consists of 0.5 mg of nicotine, which is the active pharmaceutical ingredient (API) in the product. The shelf life of NNS is 2 years, where the nicotine degrades with time to nicotine related substances (NRS). These substances will be investigated as well as the preservatives methylparaben and propylparaben. NNS is considered as a pharmaceutical product, and as for each pharmaceutical product it is critical that a validation of the used analytical method meets regulatory requirements such as Current Good Manufacture Procedure (cGMP). The requirements are fulfilled by following International Council on Harmonization (ICH) guidelines, and United States Pharmacopeia (USP) <1225> and National Formulary (NF) standards. [7]

2.2 Knowledge gap

Today, there is already existing knowledge about analysis method of NNS, yet, currently used analysis method was issued during the 20th century. The analysis method fulfilled the regulatory requirements and was therefore approved previously. However, since the requirements have been updated, also the analysis method needs to be updated to fulfil today's requirements. The method used today is divided into three methods: (1) for analysis of nicotine and preservatives called TM-005427, (2) for analysis of NRS called TM-005728, and (3) for identification, where thin-layer chromatography (TLC) is used. The third method is needed since two methods need to be applied for identification of the API.

Now, sodium dodecylsulphate is used as an ion pair reagent in reversed-phase high performance liquid chromatography (HPLC) to separate the basic analytes. By having sodium sulphonate reagents in the mobile phase, they couple with the basic compounds and form electrically neutral pairs that are retained longer in the column. To facilitate the basic compounds to form ion pairs, sodium sulphonate is diluted in an acidic mobile phase consisting of acetonitrile and water, whereas the basic analytes are ionized. Sodium acetate is used as a buffer. [8] The

columns in use during the two analysis methods (called 1 and 2 above) are µBondapak and Nova-Pak, which are suitable for a pH range of 2-8. [9]

The mentioned three currently used methods were year 2019 developed into only one method for analysis of NNS. IN this developed method, ultrahigh performance liquid chromatography (UHPLC) was applied instead of HPLC. However, this method needed further development, which is the aim of this project. The development of the method was thereafter followed with a full validation to investigate if the requirements are fulfilled. Parameters that were investigated during the development are flow, temperature, pH, and mobile phase gradient. To obtain the unprotonated species of nicotine, high pH is needed, which also wanted to be used due to practical reasons. Other analysis methods at McNeil AB used a mobile phase with a pH of 10.0, thereby it would be easier to have the same mobile phase.

As mentioned, the columns used for current methods were only suitable for pH conditions below 8, thus they were replaced with an ethylene bridge hybrid (BEH) column, where the uniform distribution of the ethylene bridge leads to a high mechanical and chemical stability of the surface. [10] Also this BEH column was used for other analysis methods, making it more practical to use. Thus, when developing the analysis method, the mobile phase and the column were a limitation since they did not want to be changed. By using those, sequences from other analysis methods can be queued after this method.

2.3 Theory

The NRS included in the study are nicotine-(cis-, trans)-N-oxide, cotinine, myosmine, ß-nicotyrine, nornicotine, methyl-3-nicotinoylpyrrolidine (MNP) and pseudo-oxy-nicotine (PON), presented in Figure 2 together with the two preservatives. Since the developed analysis method only consists of one method for all analytes, the separation between them is of high importance. (-)-Nicotine hydrogen tartrate dihydrate (NHT) is more stable than liquid nicotine when needing precise concentrations of nicotine in a solution, thus NHT was used instead of liquid nicotine during the analysis. [11]

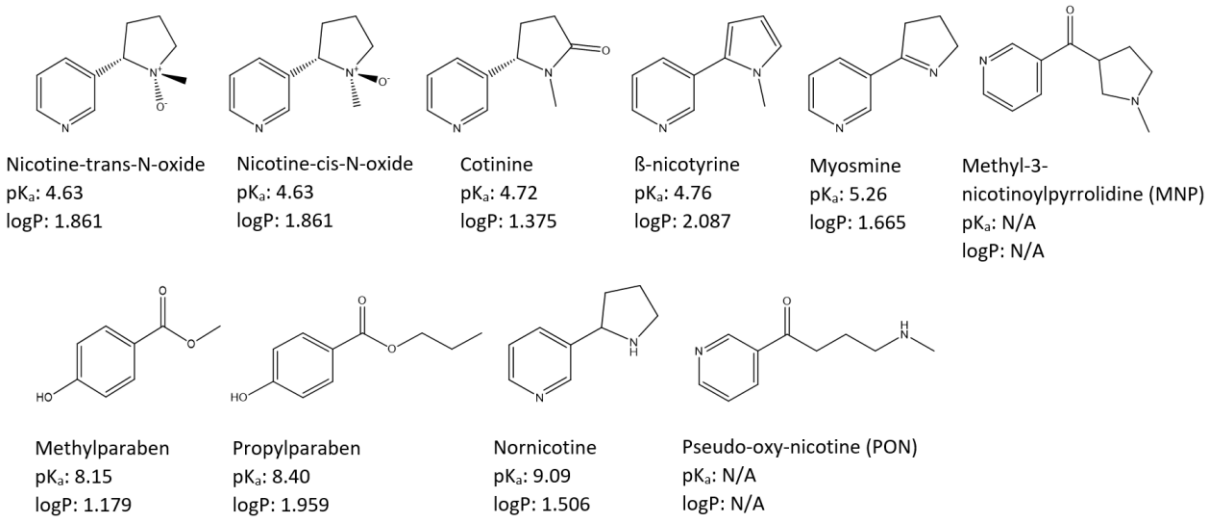

Figure 2: Molecular structure of NRSs, methylparaben and propylparaben, where logP [12] and pKa [13] are presented for each analyte.

The large variation in pK_a and logP values between the analytes require a gradient elution to obtain separation of all compounds within a reasonable time. For practical reasons as mentioned before, the organic solvent in the analysis is mainly acetonitrile. Another option is to use methanol, which do not affect the results according to previous knowledge, and which is to prefer in terms of green analytical chemistry. An advantage when using acetonitrile is however a lower backpressure in the column.

The conditions were determined to improve the resolution as much as possible, where the conditions of the existing developed method were used as initial values. When performing a full validation of a method, various performance characteristics are studied to determine if they meet the regulatory requirements of the analytical application. For each performance characteristic, there are individual requirements, which also vary depending on the analyte, i.e., for nicotine, preservatives and NRS. However, the requirements are divided into categories according to USP <1225>, where the API and preservatives are included in category I, and degradants are included in category II. The requirements for the identification tests are counted in category IV. [7] Also, filter study and column equivalence were investigated when developing the analysis method, this was done to study the influence of filters, and various column since an alternative column is good to have. Similarly, for these parameters, there were requirements, which investigated if there would be any severe effects if a filter is used or the column was changed. However, these were not included in the regulatory requirements.

Design of experiments (DoE) was applied when investigating the robustness by using Box-Behnken design, which is a three-level experimental design. The design avoids extreme points that are not typical. The number of experiments during the experimental design is calculated by using Equation 1 below, where *k* is the number of factors and *C* is replicates of the center points. [14]

$$2k(k-1)+C \quad (1)$$

2.4 Research questions

To further develop and validate the analysis method described in the theory, following questions were aimed to be answered:

- What chromatographic conditions are needed to achieve the best resolution for the analysis method?
- Is it possible to make the developed analysis method greener than currently used method?
- Are the cGMP requirements fulfilled by the developed method?

2.5 Methodology

In this study, UHPLC was used to examine and develop a new analysis method of nicotine nasal spray, where nicotine, preservatives and impurities were analysed. This was done with the aim to achieve a test method that fulfil the requirements of cGMP, which was investigated by a full validation.

3 Materials and Methods

3.1 Chemicals

The chemicals used are presented in Table 1.

Table 1: Chemicals with the quality, where they are bought from, CAS-number, and their molar masses.

Chemicals	Quality	Bought from	CAS-number	Mw [g/mol] [15]
Acetic Acid	100%, glacial	Merck, Darmstatd, Germany	64-19-7	60.05
Ammonia	25%	Merck	1336-21-6	17.03
Acetonitrile	99.9% HPLC	Labscan, Dublin, Irland	75-05-8	41.05
Purified water	Resistivity ≥18.2 MΩcm	Milli-Q	N/A	18.02
Methanol	HPLC	Merck	67-56-1	32.04
Ethanol	99.5 %	Kemetyl, Stockholm, Sweden	64-17-5	46.07

The reference standards are presented in Table 2.

Table 2: Reference standards with the quality, where they are bought from, CAS-number, and their molar masses.

Chemicals	Quality	Bought from	CAS-number	Mw [g/mol] [15]
(-)-Nicotine Hydrogen Tartrate dehydrate (NHT)	≥ 98% HPLC	LGC, Borås, Sweden	65-31-6	498.44
Nicotine-(cis-, trans)-N-oxide	-	LGC	491-26-9	178.24
Myosmine	≥ 98%	LGC	532-12-7	146.19
Cotinine	≥ 98%	LGC	486-56-6	176.22
Nornicotine	≥ 95%	LGC	494-97-3	148.21
Methyl-3-nicotinoylpyrrolidine (MNP)	98.4%	LGC	125630-26-4	190.25
Pseudo-oxy-nicotine (PON)	97%	LGC	66093-90-1	251.15
ß-Nicotyrine	≥ 95%	LGC	487-19-4	158.20
Methylparaben	99.9%	Merck	99-76-3	152.15
Propylparaben	99.8%	Merck	94-13-3	180.20

3.2 Equipment

- Dark glass bottles for storage of NRSs-solutions
- UHPLC: Waters ACQUITY-H with column oven used with PDA detector (Waters, Milford, MA, USA).
- HPLC (for method equivalence): Waters 2695 tandem UV-detector (Waters).
- Analytical column: Waters Acquity UPLC BEH C18 column, 2.1 x 100 mm, particle size 1.7 µm (Waters).
- Analytical column (for column equivalence): Acquity PREMIER BEH C18 2.1 x 100 mm, particle size 1.7 µm (Waters).
- Heating furnace
- Three various syringe filters, with pore size 0.2 µm: PES (polyether sulfone), PTFE (polytetrafluorethylene), and PVDF (polyvinylidene difluoride)

3.3 Sample preparations

Mobile phase A, 0.01 M ammonium acetate: 7.0 ml of 1 M acetic acid and 3.6 ml of 25% ammonia were mixed and then filled with purified water to 5 l.

Mobile phase B, AcN:MeOH (90:10): 50.0 ml of MeOH was added to 450.0 ml AcN.

NRS solutions: Separate NRS solutions, 1 mg/ml, where prepared by dissolving each NRS in AcN, except of PON which was dissolved in purified water. These solutions were diluted with purified water to separate, as well as combined NRS solutions of 10 µg/ml.

NIC solution, 2000 µg NIC/ml: 310.0 mg of NHT was dissolved in 50.0 ml of mobile phase A.

Preservatives' solution, 105/45 µg/ml: 70.0 mg methylparaben and 30.0 mg propylparaben were added to 10.0 ml of EtOH. The mixture was filled with purified water to 200.0 ml. 15 ml of the mixture was diluted to 50 ml with mobile phase A to achieve the right concentration.

Standard solutions: NIC, preservatives and NRS were mixed with mobile phase A to combined standard solutions with the concentrations presented in Table 3.

Table 3: Standard solutions of nicotine, preservatives and NRS.

	Nicotine [µg/ml]	Preservatives (methyl/propyl) [µg/ml]	NRSs [µg/ml]
Std 1	84	5.7/2.4	0.15
Std 2	95	6.3/2.7	0.25
Std 3	107	7.3/3.1	0.35
Std 4	115	8.4/3.6	0.70

Placebo: Sodium chloride, NNS aroma, EDTA, citric acid, sodium dihydrogenphosphour dihydrate, polysorbate and disodiumphosphate dodekahydrate were dissolved in purified water.

NNS preparation: Authentic batches from NNS production were used, however these were stressed before usage by heating each NNS bottle to 45 °C for 2 hours. The NNS solution was then diluted in mobile phase A, followed with spiking with NRS.

3.4 Method

The PDA detector was set to measure wavelengths within the range 230-310 nm. The wavelengths detected for each analyte were approximately at their respective absorbance maximum, known from previous methods to be:

λ= 236 nm: Myosmine, PON, MNP
λ= 260 nm: Nicotine, nicotine-(cis,trans)-N-oxide, cotinine, nornicotine
λ= 298 nm: Methylparaben, propylparaben, ß-nicotyrine

Initial conditions: T_{column} = 60 °C, sample temperature = 6 °C, F = 0.6 ml/min, $V_{injection}$ =10 µl, mobile phase A = 10 mM ammonium acetate diluted in water (pH 10.0), mobile phase B = AcN:MeOH (90:10). The initial gradient is presented in Table 4.

Table 4: Initial mobile phase gradient.

Time [min]	% A	% B
0	99.9	0.1
1.5	97.0	3.0
2.5	90.0	10.0
4.5	80.0	20.0
6.5	60.0	40.0
8	5.0	95.0
9	99.9	0.1
11	99.9	0.1

The temperature of the column, the flow, the gradient, and the pH of mobile phase A were adjusted to improve the resolution between the analytes. Also, the analysis time wanted to be reduced to minimize the consumption of solvent used during the analysis. Different temperatures up to 75 °C were examined, as well as the flow were increased up to 0.8 ml/min. The mobile phase gradient was mainly adjusted to increase the separation of the last eluting peaks by varying the steepness. When the flow was varied, also the gradient times were adjusted. Since the pH needed to be approximately 10.0, only small adjustments were made.

The chromatographic conditions that were examined to lead to the best resolution, and that were used further in the validation were: T_{column} = 70 °C, F = 0.8 ml/min, mobile phase A = 10 mM ammonium acetate diluted in water (pH 10.0), and the gradient as presented in Table 5.

Table 5:Mobile phase gradient for conditions used in the developed method.

Time [min]	% A	% B
0	99.9	0.1
1.2	97.0	3.0
2.2	90.0	10.0
6.5	80.0	20.0
7.5	5.0	95.0
8	99.9	0.1
10	99.9	0.1

The resolution required to be achieved between the peaks was 1.5, however, to improve the robustness of the method, a resolution of at least 2.0 was aimed to be achieved between the

NRS, and a resolution of at least 4.0 for NIC and preservatives. The higher resolution for NIC and preservatives is due to the difference in peak area between the peaks.

3.5 Validation of method

The performance characteristics included in the validation are linearity, quantitation limit (QL), accuracy, carryover, stability, robustness, precision, specificity, and method equivalence. Even though the column equivalence and filter study is not included in the validations, they will be presented in this section as well.

3.5.1 Linearity

An external calibration curve with five levels were obtained for nicotine, preservatives and NRS, where for each level, three replicates were prepared. The concentrations are presented in percentage label claim (% LC) in Table 6, where 100 %LC represents 10 mg NIC/ml.

Table 6: Concentrations for nicotine, preservatives, and NRSs for the linearity standards (LSL).

	Nicotine (% LC)	Preservatives (%LC)	NRS (% LC)
LSL 1	50	50	0.07
LSL 2	70	70	0.15
LSL 3	100	100	0.35
LSL 4	130	130	0.65
LSL 5	150	150	1.0

A linear regression was fitted, where a response factor (RF) was calculated for each data point using Equation 2. The RF values were used to obtain % RSD by using Equation 3.

$$RF = \frac{response\ of\ analyte}{concentration\ of\ analyte} \quad (2)$$

$$\%\ RSD = 100 * \frac{std(RF)}{\overline{RF}} \quad (3)$$

3.5.2 Accuracy

Three levels were studied, where the solutions were combined with NIC, preservatives and NRS. For each level, three replicates were prepared. Obtained concentrations from these were later used to calculate individual and mean recovery. The individual recovery was obtained by using Equation 4, where $c_{\text{analyte,obtained}}$ is the concentration obtained from the chromatography,

and $c_{\text{analyte,nominal}}$ is the nominal concentration. The individual recoveries for nicotine and preservatives were then used to calculate the mean recovery.

$$Recovery_{ind} = 100 * \frac{c_{\,analyte,obtained}}{c_{analyte,nominal}} \quad (4)$$

For the NRS, the individual and mean recovery was calculated for low and high level respectively, which represent 0.1 %LC and 0.8 %LC, correspondingly.

3.5.3 Specificity

The specificity was investigated using placebo, NRS mentioned in the theory, and other NRS and impurities from NIC and preservatives such as anabasine, anatabine, nor-cotinine, nicotinic acid, β-nornicotyrine, and p-hydroxybenzoic acid. The last-mentioned analyte is the main degrading product, formed from the preservatives. As compared to the other runs, the NRS were prepared separately instead of combining them in one solution. Matrix effects and spectral purity were studied for the peaks.

To identify the purity of the peaks, peak purity angel was compared with peak purity threshold, where the purity angle should be lower than the threshold for an acceptable value. The requirements for identification were to obtain positive identification of nicotine and preservatives, while negative identification for the placebo. Since the identification of analytes needs to be determined with two various approaches, both absorbance maxima and retention times were compared to a reference standard.

3.5.4 Carry over

The difference between two blanks was investigated after injection of LSL 5. An NNS batch was included in the run to calculate the difference in %LC.

3.5.5 Column equivalence

Column equivalence was performed by comparing the initial column with Acquity BEH C18 Premier 1.7 µm 100x2.1 mm. The run included placebo, NNS solution, and six replicates of NNS solution spiked with NRS. The absolute difference in %LC was calculated for preservatives and NIC between the columns, whereas the relative percentage difference was calculated for the NRS.

3.5.6 Robustness

The robustness of the method was investigated on an active NNS sample spiked with NRS by using the Box-Behnken design with the flow of the mobile phase, the pH and the temperature in the column. The adjustments made were ± ~5%, ± ~1 and, ± ~3 °C, respectively as shown in Table 7, where the runs were obtained by randomization. The table includes 15 runs, however, as mentioned in the theory, only 13 experiments would be enough to get the response surface. Yet, three center points were collected to be able to examine the variation of the center points regarding to the time.

Table 7: Box-Behnken design for flow, pH and temperature.

Runs	Flow [ml/min]	pH	Temperature [°C]
1	0.84	10.0	67
2	0.8	9.9	73
3	0.8	10.1	73
4	0.76	10.0	67
5	0.76	10.1	70
6	0.8	9,9	67
7	0.8	10.0	70
8	0.76	10.0	73
9	0.84	10.1	70
10	0.8	10.1	67
11	0.76	9.9	70
12	0.8	10.0	70
13	0.84	10.0	73
14	0.8	10.0	70
15	0.84	9.9	70

3.5.7 Precision

System precision was performed by running an SST, i.e., STD 3, including 6 injections. The area (A) obtained was used to calculate %RSD, see Equation 5.

$$\% \, RSD = 100 * \frac{std(A)}{\bar{A}} \quad (5)$$

Intermediate precision was performed by using two UHPLC-systems, columns, and analysts respectively. Each analyst prepared standard solutions and mobile phases according to the method. Also, six replicates of NNS solution spiked with NRS were prepared for each day. The same batch was used for all runs, however, different NNS flasks were used for each occasion. The arrangement for each occasion is presented in Table 8, where 1 and 2 represent the two variations.

Table 8: Procedure of intermediate precision.

Occacion	1	2	3	4	5	6
Analyst	1	1	1	2	2	2
UHPLC-system	1	1	2	2	2	1
Column	1	2	1	2	1	2
Mobile phase	1	2	2	2	1	1
Standard solutions	1	1	2	2	1	2

The investigation of intermediate precision was performed by using ANOVA. Mean square values (*MS*) within and between the groups obtained from ANOVA were used to calculate %RSD as presented in Equations 6 and 7. S_{tot} is the total variation, whereas S_w and S_b represent the variation within the groups and between the groups, respectively.

$$\%RSD = 100 * \frac{S_{tot}}{\bar{x}} \quad (6)$$

where,

$$S_{tot}^2 = S_w^2 + S_b^2 = MS_{within\ groups} + \frac{(MS_{between\ groups} - MS_{within\ groups})}{count} \quad (7)$$

The %RSD for method precision was obtained through Equation 8.

$$\%RSD = 100 * \frac{S_w}{\bar{x}} \quad (8)$$

3.5.8 Stability

The stability was investigated on NNS sample spiked with NRS, and standard solutions where vials were compared with measuring flasks, where the solutions were stored. The stability of NNS sample was investigated for 24 hours, 3 days and 1 week, whereas the standard solutions

were investigated once a week for five weeks. The absolute difference in %LC was calculated for preservatives and NIC, and the relative percentage difference for the NRS.

3.5.9 Method equivalence

The method developed during this project was compared to the two currently used methods TM-005427 and TM-005728 by preparing six NNS solutions spiked with NRS for each method. As control, also an NNS solution was prepared for each method. For identity of nicotine, both TLC and TM-005427 were used.

3.5.10 Filter study

Three types of filters were used: PES (0.22 μm), PTFE (0.2 μm), and PVDF (0.2 μm). Six replicates of NNS solution spiked with NRS were prepared for each filter type, where a non-filtered solution was used for comparison. The recovery was calculated for nicotine and preservatives for each filter, while the relative percentage difference was calculated for the NRS.

4 Results and discussions

Absorbance spectra for each analyte were obtained to visualize the absorbance maximum, presented for three analytes in Appendix A. The absorbance maxima for each analyte matched the given wavelength in the method.

Below in Figure 3, chromatograms from currently used methods are presented, which is attached to be able to visualize and compare with the results obtained from the new method.

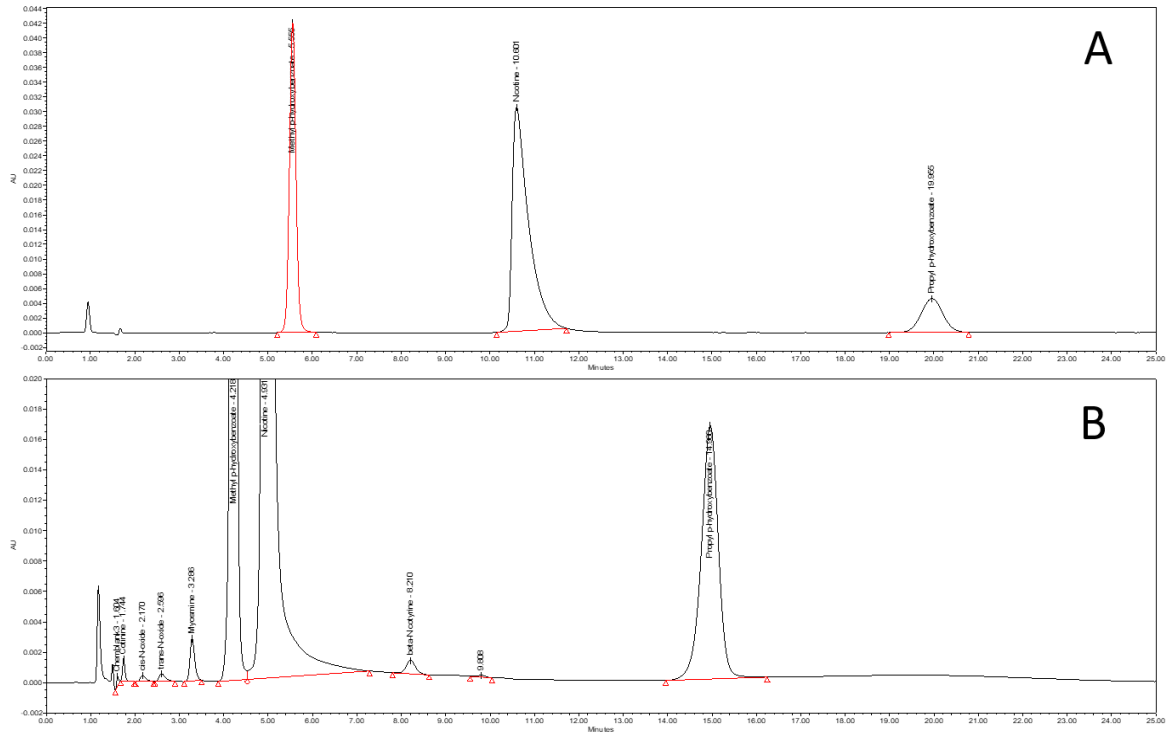

Figure 3: Chromatogram A and B representing currently used methods for nicotine and preservatives, and for NRS, respectively.

4.1 Development of analysis method

The chromatogram obtained from the initial conditions is presented in Figure 4, where also the peaks are identified. Comparing this chromatogram with the ones obtained from currently used methods, an improvement of peak resolution is clearly seen.

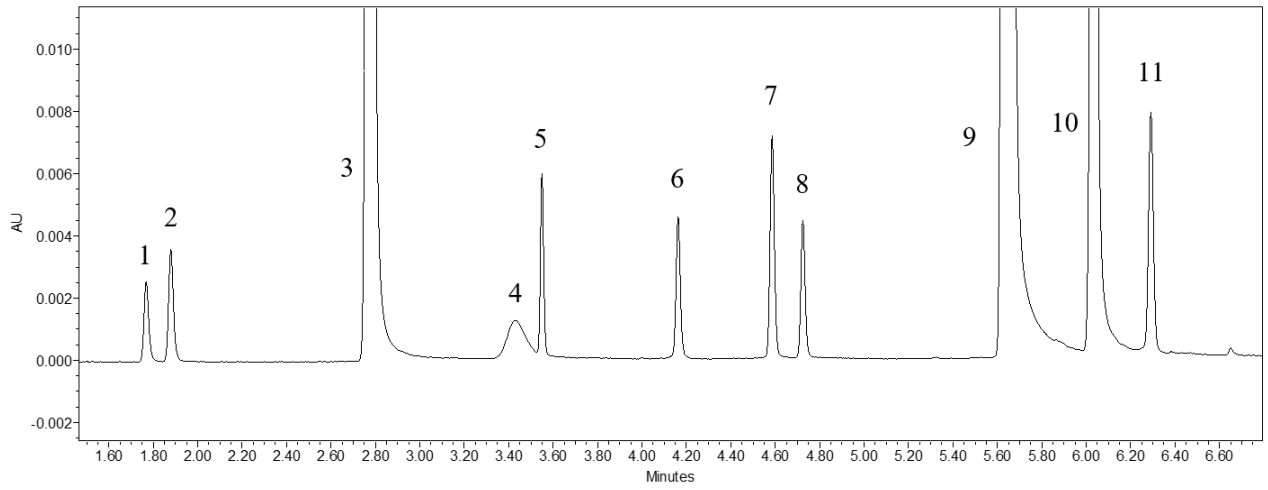

Figure 4: Chromatogram when having the initial conditions where the peaks are (1) nicotine-cis-N-oxide, (2) Nicotine-trans-N-oxide, (3) methylparaben, (4) PON, (5) cotinine, (6) nornicotine, (7) myosmine, (8) MNP, (9) NIC, (10) propylparaben, and (11) β-nicotyrine.

The initial conditions led to a resolution that fulfilled the requirement of 1.5 for all peaks except for PON and cotinine, labelled as 4 and 5 in Figure 4 respectively. Thereby, PON and cotinine were considered as critical peaks along with NIC (peak 9) during the development. Also, propylparaben and β-nicotyrine (peak 10 and 11) were included to be further investigated due to the closeness of the peaks to the API.

Initially, the flow was varied by increasing it to 0.7 ml/min and 0.8 ml/min. The flow change did not lead to any proportional variation for the peaks, leading to a change in resolution. For PON/cotinine, the separation was improved, while the opposite for the latest eluting peaks, presented in Table 9. The adjustment to 0.8 ml/min led to an increase of backpressure in the column, which was counteracted by increasing the temperature. Other solutions to decrease the backpressure could be to use a shorter column, according to Darcy's law, or larger particles in the column. However, the chosen column was practical due to other analytical methods at the company and did not want to be changed. Also, from earlier experience, when a shorter column is used, a pH of 9.8 is needed to separate PON and cotinine, which as well is not practical since a mobile phase different from the other analyzes would be needed in that case.

An increase in temperature lead as well to an improved resolution between PON and cotinine, but lower between propylparaben and β-nicotyrine. The reversed effect is due to which peaks that are mostly affected by the adjustment. A higher temperature decreases the elution time for the analytes, however the most affected peaks are PON and β-nicotyrine, leading to a better

separation for PON/cotinine, and eventually co-elution of β-nicotyrine with propylparaben. In Table 9, the resolution between the critical peaks for each adjustment is presented, where the conditions are labelled A-H. To clarify, for all runs, the initial mobile phase gradient was used.

Table 9: Resolution between PON and cotinine, NIC and propylparaben, and propylparabene and β-nicotyrine given for different flows, F, and temperatures, T. The runs are labelled from A to H.

Conditions	R_s (PON/Cotinine)	R_s (NIC/propylparaben)	R_s (Propylparaben/ β-nicotyrine)	Labeling
F=0.6 ml/min, T=60 °C	1.35	8.68	5.94	A
F=0.7 ml/min, T=60 °C	1.47	7.45	7.10	B
F=0.7 ml/min, T=65 °C	1.82	8.20	5.22	C
F=0.7 ml/min, T=70 °C	2.14	8.13	3.49	D
F=0.8 ml/min, T=70 °C	2.66	9.54	Co-elution	E
F=0.6 ml/min, T=75 °C	2.45	9.90	Co-elution	F
F=0.7 ml/min, T=75 °C	2.51	9.12	Co-elution	G
F=0.8 ml/min, T=75 °C	2.62	8.17	1.70	H

Important to be aware of is that not all conditions were analyzed during the same day, meaning that different mobile phases were prepared for several runs. As will be discussed further, the analysis is sensitive to small changes in pH. By using different mobile phases, small differences in pH will appear, which may have led to misleading results. This is assumed to be the case for run E, where the resolution for PON/cotinine is expected to be lower than for run H. Also, even for the runs where the same mobile phase was used, the pH altered from day to day.

To summarize, the increase in temperature and flow improved the separation between PON and cotinine, while the opposite between propylparaben and β-nicotyrine. It was decided to further investigate conditions C, D and E, with the aim to improve the resolution between the latter peaks by varying the mobile phase gradient. The less steep gradient, the higher resolution is obtained, observed for all three conditions according to

Table 10 below. The gradient for run C was adjusted to 35 % and 30 % acetonitrile at 6.5 min, presented as C1 and C2 respectively. In run D, the percentage acetonitrile was decreased to 30 (D1) and 20 (D2) at 6.5 min, and in the same manner, the gradient was adjusted to be less steep for run E. Yet in run E, also the times for the gradient were changed. In run E1, the acetonitrile

increased from 10 % to 20 % between 2.2 and 6 min, while for E2 the increase was between 2.2 and 6.5 min.

Overall, the adjustments show that all conditions may result in the aimed resolutions by making small modifications of the gradient. However, with the resolution of PON/cotinine included during selection, the conditions of interest were 0.8 ml/min and 70 °C. The gradient for these conditions that was most suitable was E2, where the robustness of the method was increased due to good separation between all peaks.

Table 10: Resolution between NIC/ propylparaben, and propylparaben/ β-nicotyrine for three conditions, given for the initial mobile phase gradient together with two additional gradients.

	C	C1	C2
F=0.7 ml/min, T=65 °C	8.20 / 5.22	7.20 / 6.16	7.16 / 6.33
	D	**D1**	**D2**
F=0.7 ml/min, T=70 °C	8.13 / 3.49	7.66 / 3.86	6.26 / 5.45
	E	**E1**	**E2**
F=0.8 ml/min, T=70 °C	9.54 / co-elution	6.21 / 4.45	6.02 / 4.67

Another thing observed when increasing the flow is the improvement in peak shapes, where overall, tailing was reduced, and the peak widths are smaller. The improved peak shapes are due to less time is spend in the column with an increased flow. A higher flow leads to less longitudinal diffusional, giving lower plate height (*H*), which result in sharper peaks. However, it is important to have in mind that the plate height also is affected by the multiple paths and the mass transfer resistance, where the mass transfer is increased with higher flow according to van Deemter equation, see Equation 9. The pursuit is a fast diffusion in radial direction to counteract the spread of molecules, with various and short distances from the stationary phase, affected by the flow. A, *B*, and *C* are three constants for a given column and stationary phase, and u_x is the linear velocity, proportional to the flow rate. [16]

$$H = \underbrace{A}_{\text{Multiple paths}} + \underbrace{\frac{B}{u_x}}_{\text{Longitudinal diffusion}} + \underbrace{Cu_x}_{\text{Mass transfer}} \qquad (9)$$

The improved peak shapes lead in general to more accurate quantitation, and improved resolution, described from Equation 10, where Δt_r is the difference in retention time between two peaks, and w_{av} is the average width of the two peaks. [16]

$$R_s = \frac{\Delta t_r}{w_{av}} \quad (10)$$

Finally, the pH was adjusted. Already when developing the other parameters, it was understood that the pH had a great effect on the resolution, which is because the separation factor is highly dependent on the degree of analyte ionization. It was observed that the more acidic the mobile phase was, the better resolution between PON and cotinine, which is due to the earlier eluting PON with lower pH. Yet, the decrease in pH lead to later elution of the preservatives. Too low pH would therefore lead to co-elution of PON and methylparaben, since they move against each other, and eventually, propylparaben will co-elute with β-nicotyrine. When the pH is increased instead, the opposite appearance will occur, i.e., PON elutes later, leading to co-elution with cotinine, while propylparaben elutes closer to nicotine. In Figure 5, chromatograms from four different runs with various pH are attached.

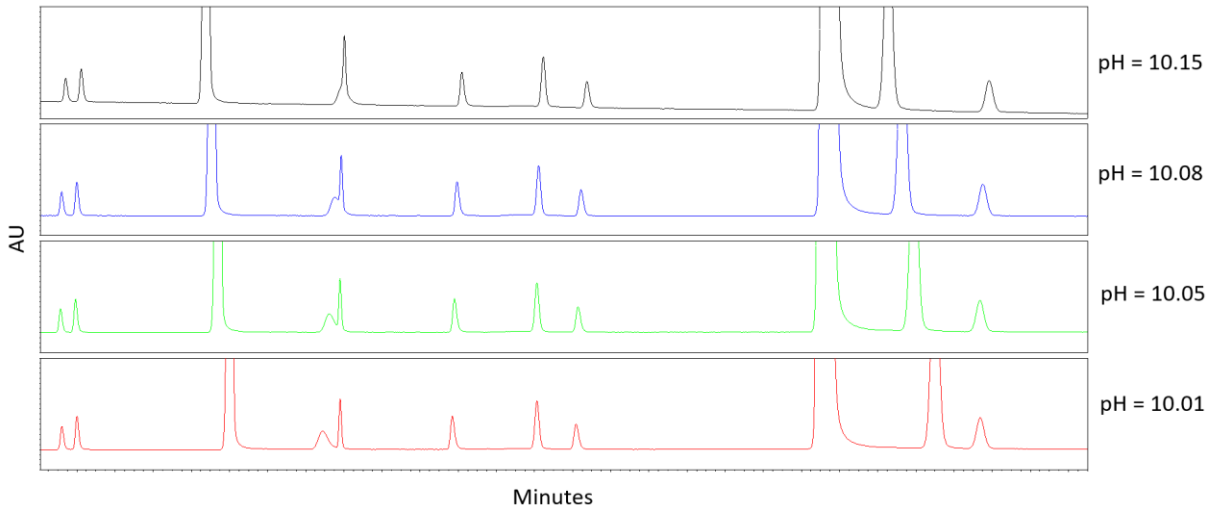

Figure 5: Four chromatograms with various pH of the mobile phase.

Since such small variations control the resolution between the critical peaks, it is required to run some pre-injections before the analysis to ensure good resolution. Another reason why pre-injections are needed is due to the pH cannot be identified with more than 0.05 assurance, which as discussed is enough to impact the chromatograms. One thing observed when preparing the mobile phase was the influence of the ammonia solution, where a new opened bottle resulted in moderate pH, while an older ammonia bottle led to more adjustments needed to obtain an acceptable resolution. The chromatogram obtained when using the conditions used in the developed method is presented in Figure 6.

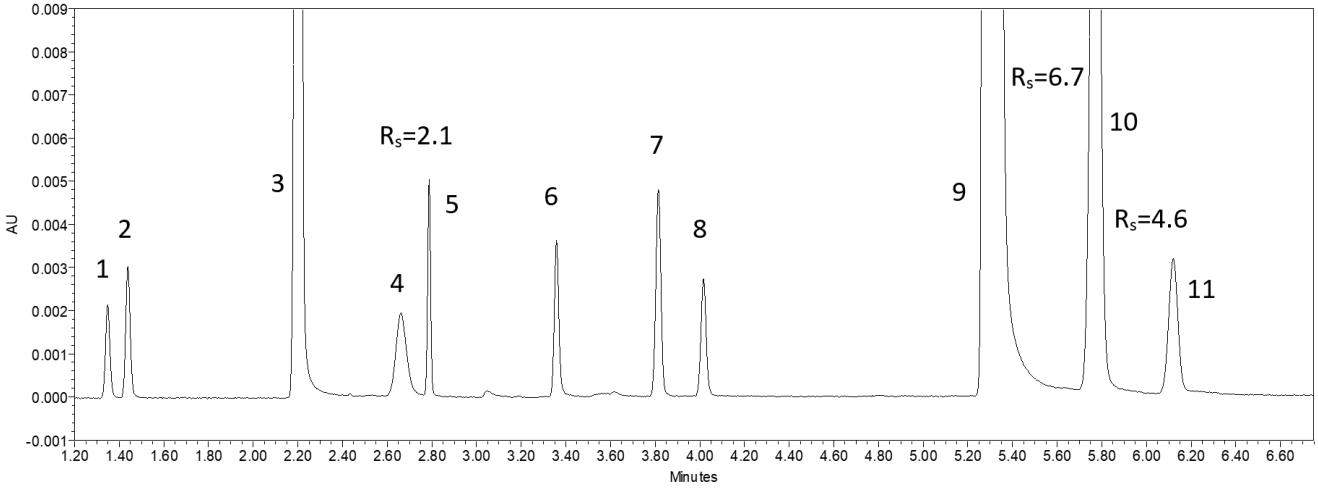

Figure 6: Chromatogram when having the conditions used for the developed method: 0.8 ml/min, 70 °C, pH 10.0 and less steep gradient. The peaks are labelled with numbers as (1) nicotine-cis-N-oxide, (2) Nicotine-trans-N-oxide, (3) methylparaben, (4) PON, (5) cotinine, (6) nornicotine, (7) myosmine, (8) MNP, (9) NIC, (10) propylparaben, and (11) β-nicotyrine. The resolution (R_s) is presented for the critical peaks.

After several runs a new peak showed up in the chromatograms between nornicotine and myosmine. This peak was assumed to be a degradant from nicotine, which appears due to the high concentrations used during the analysis. The degradant also showed to have similar absorbance spectra as nicotine. It was observed that the peak increased with higher concentration of nicotine when studying the standards, and as expected, the peak became distinctly larger in the chromatogram of a sample injection. In Figure 7, the degradant elutes between nornicotine and myosmine. To eliminate this peak, the system was washed with phosphoric acid, followed by a wash with methanol and lastly purified water. An alternative to phosphoric acid is to wash the column with diluted acetic acid.

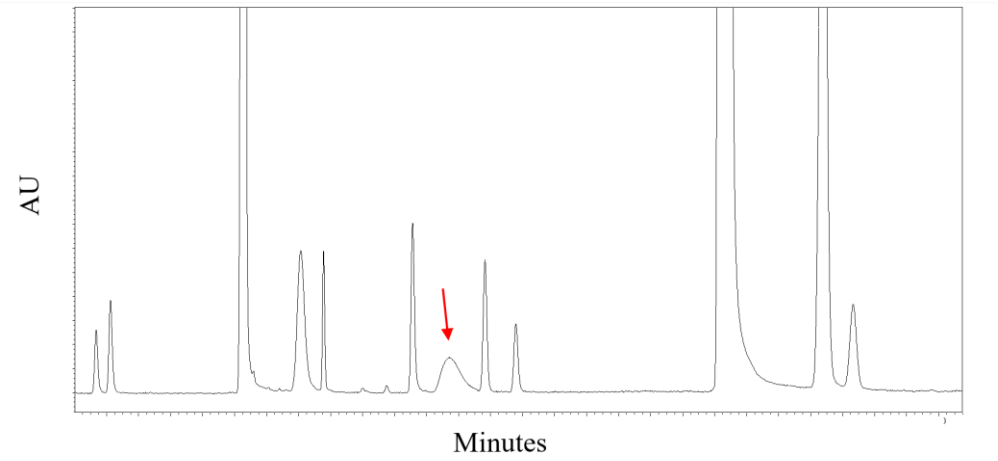

Figure 7: Chromatogram of a spiked sample, where the arrow shows an excipient.

4.2 Method equivalence in terms of green chemistry

The analysis time for the new, developed method is 10 min, with a flow of 0.8 ml/min, meaning that 0.7 ml of AcN is used for each injection. Thus, in total, approximately 9.1 ml of organic solvent is used for a run including a blank, SST, standards, a control sample, and a sample solution. Currently used methods have an analysis time of 25 min each, with the exception that the method for NRS has an analysis time of 40 min for sample injections. These methods use single point calibration but have approximately as many injections due to duplicate injections for standards, control sample and sample solutions. With a higher flow of 2 ml/min and longer analysis times, approximately 374 ml of AcN is used for each run. Thus, by using the new method, the consumed organic solvent is 40 times less than for current methods, leading the new method to be significantly greener. Appendix B presents the calculations.

4.3 Validation

Various requirements for each characteristic parameter need to be fulfilled by nicotine, preservatives, and the eight mentioned NRS. To simplify the following section, only the highest, and/or lowest, values for preservatives and NRS are presented and compared to the requirements, if the given value fulfil the requirements, the remaining analytes are accepted as well. However, one of the NRS is presented deeply together with one preservative and nicotine. It was decided to focus on nicotine-trans-N-oxide since it is the first NRS close to another NRS (nicotine-cis-N-oxide), and methylparaben since its peak is larger than propylparaben.

4.3.1 Linearity

The results obtained fulfil the requirements, as can be seen in Table 11. The linear regression for the analytes is attached in Appendix C, however, the standardized residuals for nicotine, methylparaben, and nicotine-trans-N-oxide is presented in Figure 8. The distribution of the residuals is considered to be random around zero.

Table 11: Requirements and results presented for linearity.

	Acceptance criteria	Requirement	Result
NIC	r^2	≥ 0.999	1.00
	% RSD	≤ 3.0	0.22
Preservatives	r^2	≥ 0.995	≥ 1.00
	% RSD	≤ 4.0	≤ 0.36
NRS	r^2	≥ 0.99	≥ 1.00
	% RSD	≤ 15.0	≤ 3.49

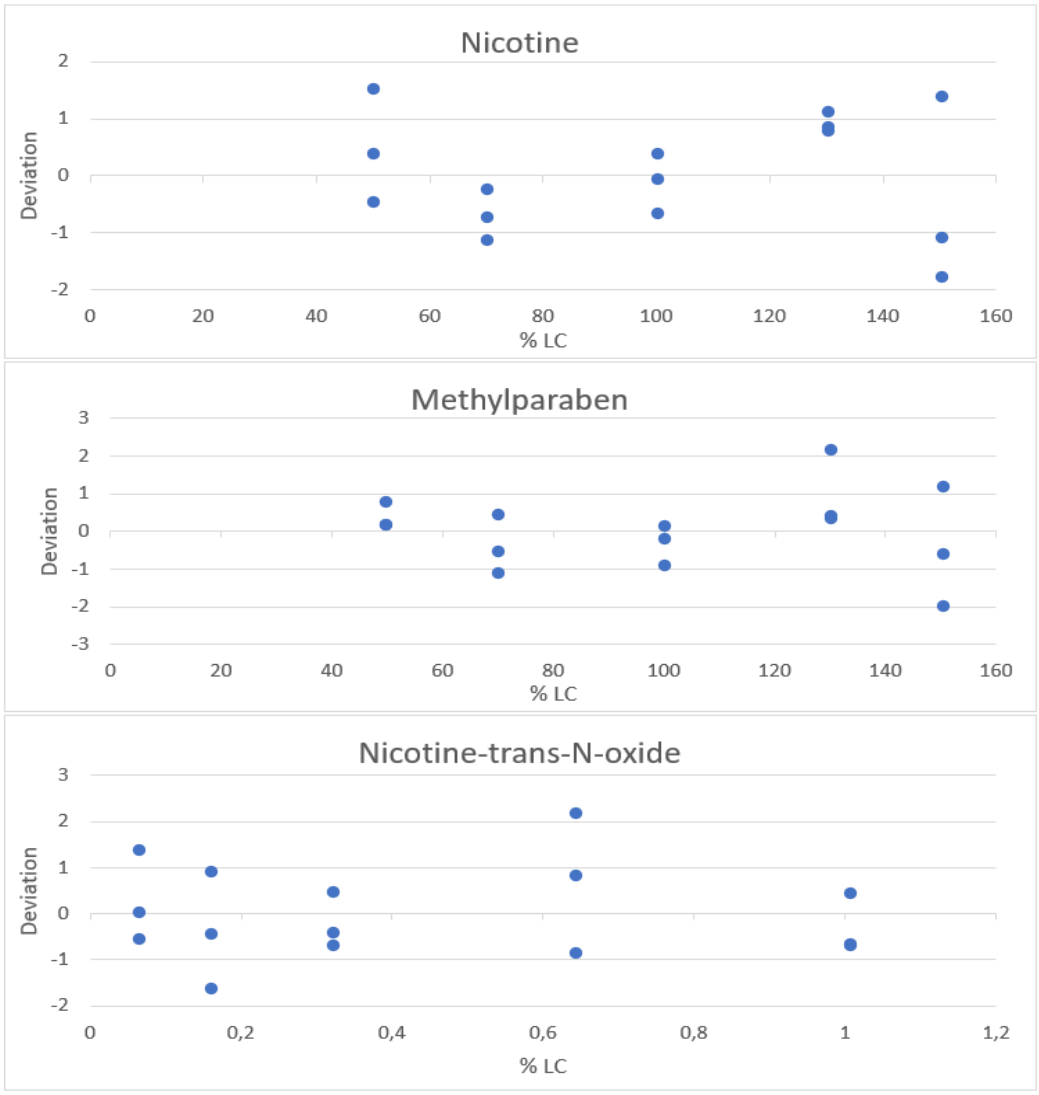

Figure 8: Standardized residuals vs % LC for nicotine, methylparaben, and nicotine-trans-N-oxide, respectively.

4.3.2 Accuracy

Overall, high recovery was obtained for all analytes. The results and the requirements are presented in Table 12 after subtraction from 100%, where both individual and mean recovery are included. The individual recoveries that are presented are the most extreme values. For the NRS, low and high levels were presented separately due to various requirements.

Table 12: Requirements and results presented for accuracy.

	Acceptance criteria	Requirement	Result
NIC	% Recovery, Individual	± 3	Nmt ± 0.5
	% Recovery, Mean	± 2	+ 0.2
Preservatives	% Recovery, Individual	± 5	Nmt ± 0.3
	% Recovery, Mean	± 3	Nmt ± 0.07
NRS	% Recovery, Individual – Low level	± 50	Nmt ± 5.9
	% Recovery, Individual – High level	± 30	Nmt ± 0.89
	% Recovery, Mean – Low level	± 20	Nmt ± 3.9
	% Recovery, Mean – High level	± 10	Nmt ± 1.1

4.3.3 Specificity

The peaks detected from the placebo, did not affect the quantification, thus no matrix effects were obtained, see Table 13. For all peaks but myosmine, the purity angle is lower than the purity threshold, meaning that myosmine is the only analyte with lower purity than the requirements. That is the case when studying the chromatogram at 260 nm. The reason to the non-pure myosmine peak is the appearance of the earlier mentioned degradant from nicotine, which during the specificity run appears below myosmine. An explanation to the change in retention time of the degradant is the influence of pH. Yet, when changing the wavelength to 236 nm, myosmine is not affected by the degradant due to myosmine is approximately 3 times larger and the degradant is 3 times smaller at 236 nm than at 260 nm.

Table 13: Requirements and results presented for specificity.

	Acceptance criteria	Requirement	Result
NIC	Matrix effects, %LC	≤ 0.1	Not detected
Preservatives		≤ 0.5	Not detected
NRS		≤ 0.05	Not detected

The other NRS and impurities from NIC and preservatives did not disturb any peaks or co-elute with any of the analytes included in the analysis method. Even if these additional NRS and impurities was not found in the sample solution, it was crucial to investigate possible co-elution since they may be found in the raw material. Nicotinic acid is the first eluting peak, which is expected for an acid during the basic conditions. The last eluting NRS of interest is known to be β-nicotyrine from previous methods, making it possible to have such flat gradient. Also, another run with a longer gradient in the end was investigated, showing matrix effects after elution of β-nicotyrine. The peaks could be separated but was not of interest. By knowing that β-nicotyrine is the last eluting peak of interest, it is possible to start the washing right after. Identification of nicotine and preservatives were positive by using both retention time and absorbance maxima.

4.3.4 Carry over

The calculations from the carry over is presented in Table 14, where all analytes fulfil the requirements. The small values mean that the interference of the small peaks obtained from the blank is neglectable and do not disturb the analyte sample.

Table 14: Requirements and results presented for carry over.

	Acceptance criteria	Requirement	Result
NIC	Any, interference, % LC	≤ 0.1	0.006
Preservatives		≤ 0.5	≤ 0.03
NRS		≤ 0.05	≤ 0.005

4.3.5 Column equivalence

As presented in Table 15, the results for nicotine and preservatives fulfil the requirements, meaning that the change of the column did not have any severe effects. However, PON and nornicotine among the NRS resulted in a large variation in concentration between the columns,

giving a relative percentage difference of approximately 50.0, not fulfilling the requirement with a limit of 30.0.

Table 15. Requirements and results presented for column equivalence.

	Acceptance criteria	Requirement	Result
NIC	% RSD	≤ 2.0	0.6
	Absolut difference between BEH and premier column	≤ 3.0 %	≤ 1.4
Preservatives	% RSD	≤ 3.0	≤ 1.0
	Absolut difference between BEH and premier column	≤ 5.0 %	≤ 1.2
NRS	% RSD	≤ 20.0	≤ 12.4
	Relative percentage difference between BEH and premier column	≤ 30.0	≥ 30.0

Since the samples used were spiked with 0.2 %LC, the expected concentrations of PON and nornicotine obtained from the chromatograms should be close to that value. That was the case for the premier column but not when using the initial BEH column, where the concentrations were 0.60 %LC and 0.42 %LC for PON and nornicotine, respectively. It was thereby concluded that the two mentioned NRS were formed in the BEH column, which was assured by obtaining a concentration of 0.46 %LC and 0.24 %LC for PON and nornicotine in the sample not spiked with NRS, where the concentration should be 0 %LC. One possible explanation for the formation of those peaks is the reaction of nicotine with metals in the LC-system. This may e.g., occur in the injection needle, or in the column, leading to oxidation. By using the premier column where hybrid organic-inorganic silica is used to block interactions between analyte and metal, also the metal in LC surfaces is more protected from corrosion, which is an advantage both for the system, but also since corrosion may increase the analyte degradation. Other factors that have been confirmed to have an impact on the oxidation of amine compounds are the use of high pH of the mobile phase, and the use of certain organic solvents such as acetonitrile. [17]

However, the premier column was of interest to investigate because of the difference in metal ions. Optional would be to use a similar column from another manufacturer as an alternative, which probably would fulfill the requirements of having an alternative column.

4.3.6 Robustness

According to the Table 16, all runs fulfilled the requirements except 11 and 15. However, that is also the case for run 2 and 10, explained further down. Several runs resulted in a resolution below 1.5. The outcome is expected, since the method showed to be sensitive to small changes in pH during the development of the analysis method. Yet even though the requirement of resolution is 1.5, it was decided that 1.2 also was acceptable since the QL sample at 0.1 %LC was quantified with the same resolution. Thereby, the quantification of the NRS should be good as well.

As discussed in the development section earlier, and as should be expected, the peaks affected by the condition changes are the critical peaks but nicotine, where the resolution fulfilled the requirements in all runs. The runs that lead to the worst results were 2, 10, 11 and 15. The lower pH and higher temperature in run 2 caused a reversed elution order of propylparaben and β-nicotyrine, with a resolution of 1.1, see Figure 9. Runs 11 and 15, with low pH as well, resulted in later elution of β-nicotyrine, leading to co-elution with propylparaben, thus no values presented in the table below. It is clearly seen that the elution of the peaks is faster when increasing the flow in run 15 to 0.84 ml/min compared to run 11 with 0.76 ml/min, however, the flow does not affect the resolution between propylparaben and β-nicotyrine. Finally, run 10 with a more basic pH and lower temperature lead to a resolution of only 1.0 for PON/cotinine, which is expected due to both higher pH and lower temperature give a later eluting PON.

Table 16: Requirements and results presented for robustness for each run. Nicotine and preservatives are presented as absolute difference between current and new conditions, while the NRS is presented as relative percentage difference between current and new conditions.

	NIC	Preservatives	NRS
Requirements / Run	≤ 3.0 %	≤ 5.0 %	≤ 30.0
1	0.13	≤ 0.12	≤ 15.6
2	0.19	≤ 0.73	≤ 13.7
3	0.16	≤ 0.15	≤ 8.30
4	0.01	≤ 0.04	≤ 11.6
5	0.21	≤ 0.036	≤ 9.22
6	0.33	≤ 0.12	≤ 5.13
7	0	≤ 0	≤ 0
8	0.067	≤ 0.067	≤ 17.6
9	0.040	≤ 0.32	≤ 3.47
10	0.16	≤ 0.63	≤ 7.46
11	0.043	≤ 1.20	-
12	0.023	≤ 0.12	≤ 8.55
13	0.098	≤ 0.12	≤ 10.5
14	0.031	≤ 0.094	≤ 10.0
15	0.38	≤ 1.7	-

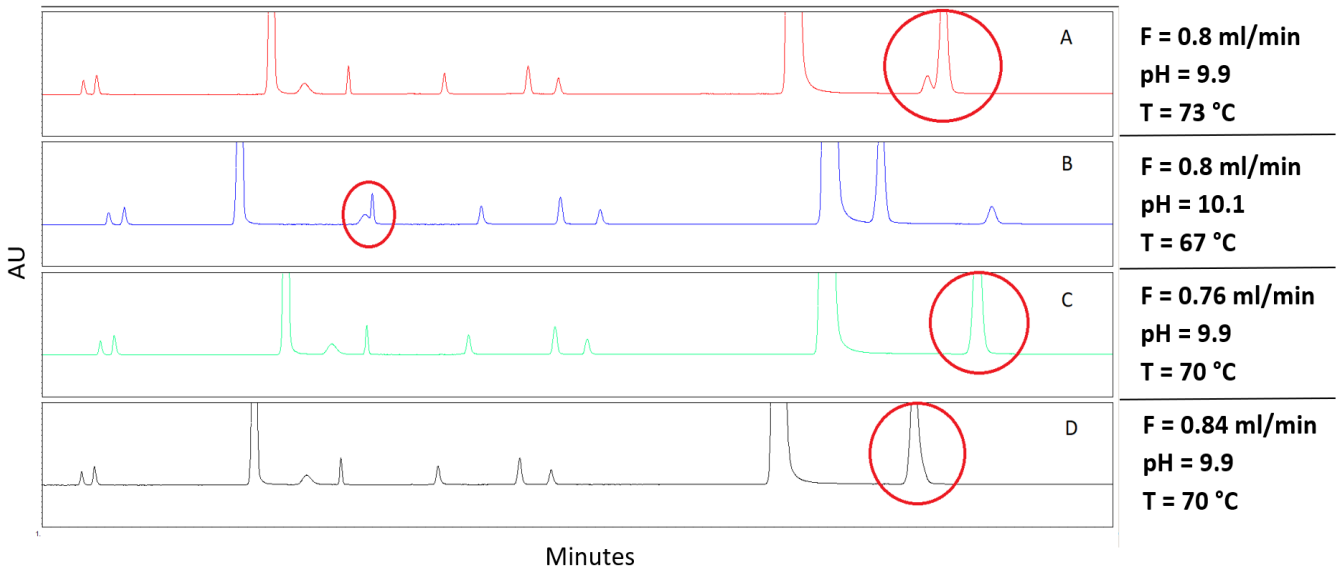

Figure 9: Chromatograms A, B, C and D represent runs 2, 10, 11, and 15, respectively. The red circles present the deviations.

By using external calibration, and thereby having all analytes included in the standards, it is possible to discover low resolution in early stage by starting the analysis with some pre-injections. Thus, by using a standard, immediate adjustments may be done until the desired resolution is obtained. If relative RF were used instead, it would not be possible to discover low resolution until after the analysis. The use of external calibration is a good advantage for the robustness since the method is influenced by small changes. Another advantage with external calibration is that each analyte is quantified by using its own calibration curve compared to if they were quantified by using the calibration curve of nicotine instead.

4.3.7 Precision

The requirements of %RSD for each analyte are fulfilled according to the results presented in Table 17. The high value of the intermediate precision for NRS, represents the total variation as a relative value for PON, where also nornicotine resulted in a high value just below PON. The occasions with higher values were 1, 5, and 6, where both columns were used, thus the variation between these occasions and the rest should not depend on which column that is used. One thing in common for the three occasions, and differencing from the others, was the used mobile phase, which was more basic than the other one, resulting in lower resolution between PON and cotinine. The higher concentrations obtained for those occasions could therefore depend on impact from the nearby peak. However, it was observed that the variance for the mentioned occasion was smaller than for the others, resulting in high total variation.

Table 17: Requirements and results presented for precision.

		Acceptance criteria	Requirement	Result
System precision	NIC	% RSD	≤ 1.0	0.20
	Preservatives		≤ 1.5	≤ 0.18
	NRS		≤ 5.0	≤ 1.5
Method precision	NIC	% RSD	≤ 2.0	0.74
	Preservatives		≤ 3.0	≤ 0.68
	NRS		≤ 10.0	≤ 2.13
Intermediate precision	NIC	% RSD	≤ 3.0	0.89
	Preservatives		≤ 5.0	≤ 1.1
	NRS		≤ 15.0	≤ 10.7

By comparing system and method precision, it was concluded that the increase in method precision is due to pipetting and dilution. When eliminating these from the intermediate precision, the total variance is still high which also probably depend on pipetting and dilution variance. However, it is expected to have higher variance between the occasions with several variating factors, than within a group.

Below in Figure 10, ANOVA can be studied visually for nicotine, where it can be seen that the standard deviations between the occasions are not significantly different.

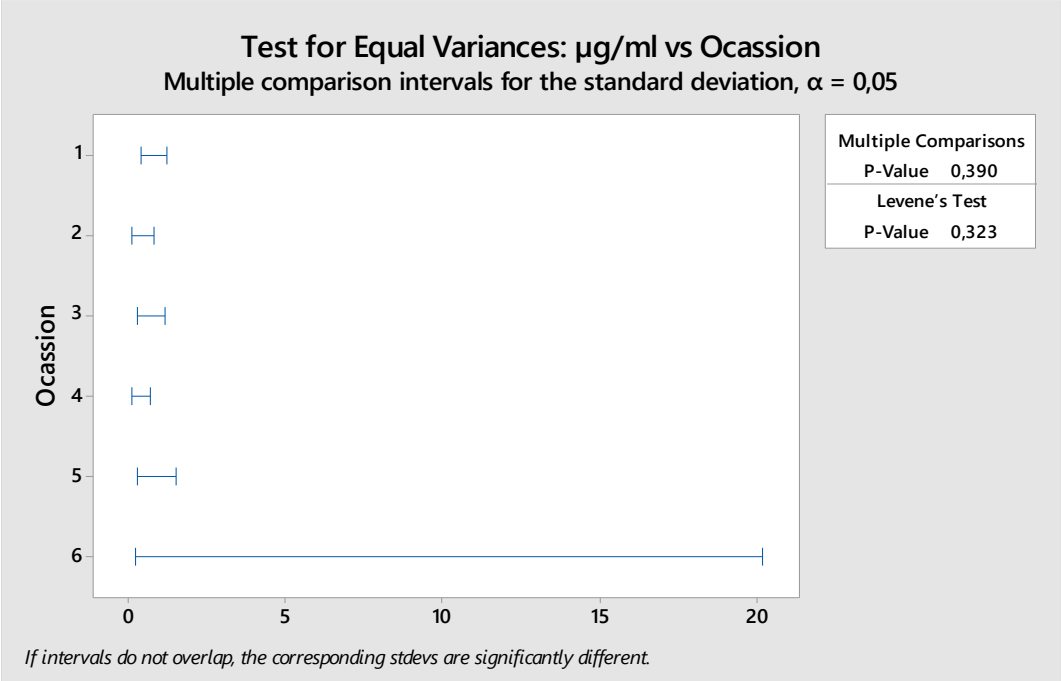

Figure 10: Variance within and between the occasions.

It is clearly seen that the variance within occasion 6 is larger than for the other occasions, which is due to a smaller concentration for one of the six prepared samples. The variation is assumed to be due to inaccuracy of pipetting, where the deviating sample can be identified in Figure 11, outside the 95% confidence interval. The inaccuracy when diluting sample 6 was confirmed by also having a lower concentration of preservatives in the same sample. No deviations were considered for the NRS in the sample, which was expected due to the separate addition of these analytes. Residuals were examined and showed that the deviating data point was not an outlier and was therefore not eliminated. Probably, the point was not considered as an outlier since there in general is a large variation in occasion 6. Yet even when having such variation, the requirements were fulfilled.

Another thing observed in Figure 11 is the variation of the mean values for each occasion from the general mean concentration, as well as the variation between the groups. Since the variance within the groups, i.e., the method precision, is low, the confidence intervals are small, which assure that the obtained value represent a more accurate value, but which also make it easier to identify any outliers. However, when having such small confidence intervals, only small variations will control if there is a significance difference from the mean value or not. Calculated from the total variance from the intermediate precision, the margin of error for the method when quantifying nicotine is ±1.8 % (95 % CI), which is less than the requirements for an individual accuracy result (±3.0 %).

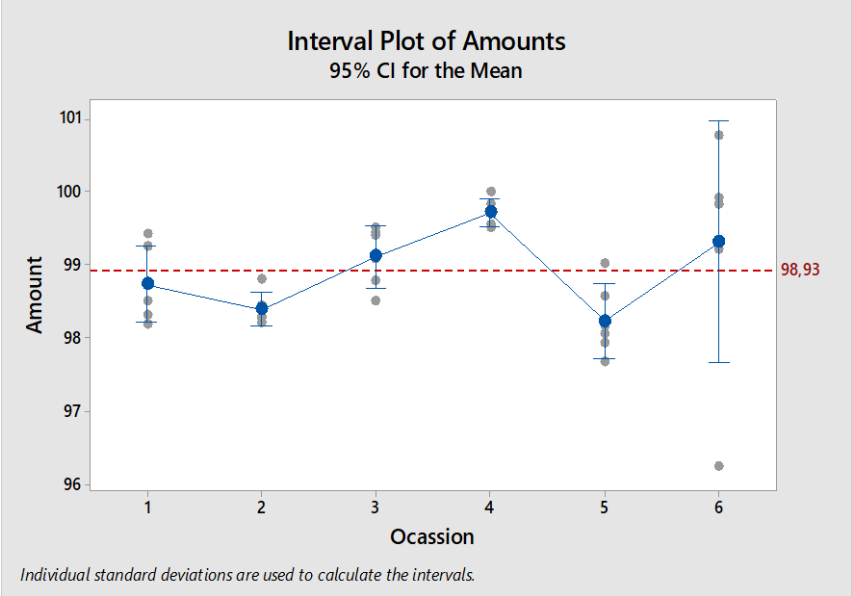

Figure 11: The variation for each group with 95 % confidence interval. The amount is given in %LC. The red line shows the grand mean value.

4.3.8 Stability

The standard solutions showed to be stable for five weeks, as shown in Table 18. To clarify, the results for week 1 represents one week after the initial standards were prepared. The presented values in the table represent the standard solution with the highest value, meaning that if the requirements are fulfilled by the given value, the other standard solutions are acceptable as well.

Table 18: Requirements and results presented for stability of standard solutions, where nicotine and preservatives are presented as absolute difference between initial and new standards.

	Requirement	Result after respective week			
		1	2	3	4
NIC	≤ 3.0 %	≤ 1.8	≤ 0.94	≤ 0.99	≤ 1.1
Preservatives	≤ 5.0 %	≤ 2.4	≤ 2.2	≤ 2.0	≤ 2.3

The NRS are not included in Table 18 due to no requirements for the standard solution. Yet, the NRS are involved when studying the stability of the sample. The requirements for nicotine and preservatives are equal to the standard solutions, while the limit of the relative percentage difference for NRS is 30.0. The sample was proved to be stable for 45 h, where PON and nornicotine limited further stability. The values calculated for PON and nornicotine after additional hours were higher than the acceptance criteria, which is due to formation of the analytes in the system as described in the column equivalence section.

4.3.9 Method equivalence

The requirements were fulfilled for all analytes, as presented in Table 19. As mentioned in the specificity, the identification of nicotine and preservatives was positive, which also was the case when comparing to currently used method and TLC. However, that is not a surprise since the samples used during this study are already released batches. The advantage with UHPLC in the new method is the increased chromatographic performance, leading to better separation of the analytes than when using TLC, thus it becomes easier to compare the retention time with a reference standard.

Table 19: Requirements and results presented for method equivalence.

	Acceptance criteria	Requirement	Result
NIC	% RSD	≤ 2.0	0.15
	Absolute difference between current and new method	≤ 3.0 %	2.9
Preservatives	% RSD	≤ 3.0	≤ 0.15
	Absolute difference between current and new method	≤ 5.0 %	≤ 1.4
NRS	% RSD	≤ 20.0	≤ 3.54
	Relative percentage difference between current and new method	≤ 30.0	≤ 17.8

4.3.10 Filter study

The recovery calculated for nicotine and the preservatives is presented after subtraction from 100 %, seen in Table 20, thereby the values represent the variation each filter resulted in. All analytes fulfilled the requirements for each filter, meaning that each of the three filters may be used. However, it was no need to filter the samples since the NNS solution is a transparent liquid with no participation.

Table 20: Requirements and results presented for filter study.

	Acceptance criteria	Requirement	Result		
			PTFE	PES	PVDF
NIC	Recovery	≤ 2.0 %	0.25	0.38	0.58
Preservatives	Recovery	≤ 5.0 %	≤ 1.1	≤ 2.4	≤ 0.66
NRS	Relative percentage difference between filter and control	≤ 30.0	≤ 3.25	≤ 10.8	≤ 8.89

4.3.11 Advantages of new developed method

There are several advantages when comparing the new method with currently used method. Of high importance is the decreased complexity and increased efficiency by replacing several methods into only one method, and by having shorter analysis time, also leading to a greener method. By changing from the used columns and the ion pair reagent to the commonly used

BEH column and mobile phase at the company, the analysis of NNS becomes more practical. The use of external calibration leads to more accurate results than when using single point calibration as in present methods, this by reducing the confidence interval when quantifying. In general, the accuracy and repeatability are improved due to higher requirements.

5 Conclusions

To conclude, the chromatographic conditions leading to the best resolution in the analysis method is: T = 70 °C, F = 0.8 ml/min, pH = 10.0, and a gradient increased to 20 % acetonitrile at 6.5 minutes. All cGMP requirements are fulfilled by the analysis method except for the robustness, where four combinations led to too low resolution. The resolution may however be inspected with some pre-injection before running the analysis. Also, column equivalence did not fulfil the requirements, where the issue leading to failure also led to very short stability of the sample, thus both these should be further investigated. Due to higher requirements, the new method has higher accuracy and repeatability, which also is less complex since the current methods were replaced with only one method. By replacing HPLC and TLC with UHPLC, the efficiency is improved, leading to that the consumed amount of acetonitrile is decreased with more than 40 times, thus the developed analysis method is greener than the present methods.

6 Future aspects

The stability of the sample, and the column equivalence should be investigated further, where both these were highly influenced by the formation of PON and nornicotine. More runs should be implied to achieve the requirements of column equivalence, and one should investigate if the sample may be stable for longer time. Another future aspect is to investigate the washing sequence, where in this method, the gradient is rapidly increased up to 90 % acetonitrile. It might however not be required to increase to 90 % to elute the remaining peaks. If the same slope of the gradient is used, but e.g., only increasing to 70 % acetonitrile, the gradient will also decrease to 0 % faster, causing the peaks to elute faster and thereby having a shorter washing sequence. Also, in the developed method, a new injection starts after the gradient have returned to 0 %. Since the injection cycle takes a while, the gradient may also be adjusted so the injection starts during the decrease of the gradient, thus the system will not pump solvent unnecessarily during the last minutes. This is possible to implement especially since there are no analytes of interested eluting before 1 min. Yet, it should be considered that the time for the injection sequence vary between different LC-systems. Developing the gradient probably will lead to even greener analysis method.

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8 Appendix

8.1 Appendix A

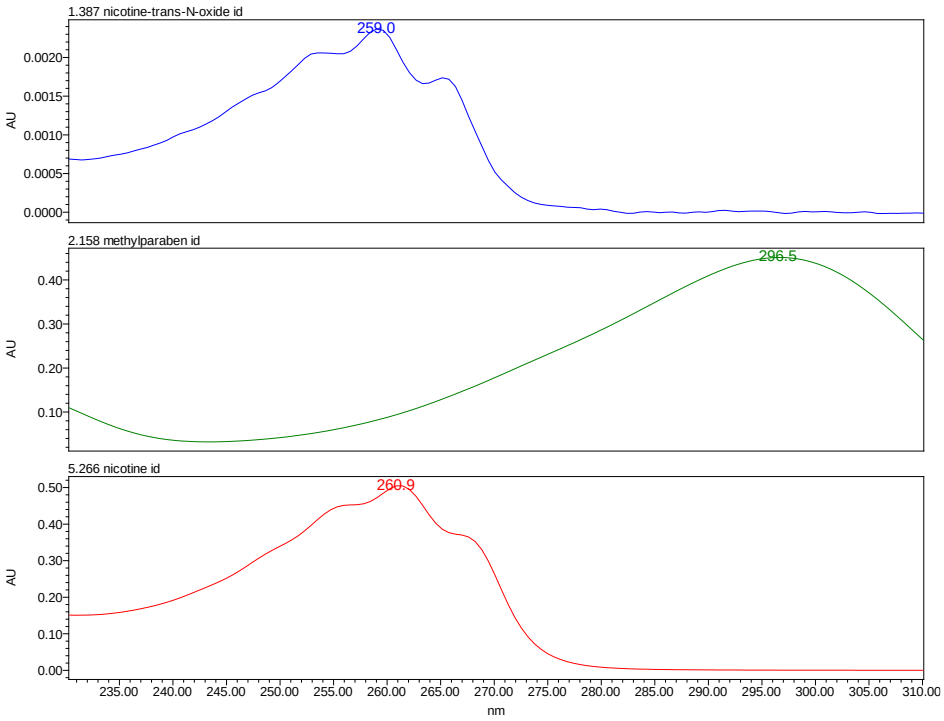

Figure A1: Absorbance spectra for nicotine-trans-N-oxide (blue curve), methylparaben (green curve), and nicotine (red curve) where the absorbance maxima is labelled with representing wavelength.

8.2 Appendix B

Calculations of how much organic solvent is consumed for one analysis sequence, including SST (6 injections), blank, standards, control sample, and sample solution.

For **present methods**, one-point calibration was used, meaning only one injection as standard. However, the method for nicotine and preservatives uses duplicates of standard, control sample, and sample solution, leading to a total of 13 injections. Each injection takes 25 min, thus the total sequence takes:

$$t = 13\ injections * 25 \frac{min}{injection} = \ 325\ min$$

The method used for NRS only use duplicates of standard and sample solution, thus 12 injections are included. The injections for the sample solution take 40 min, whereas the rest take 25 min, leading to a total analysis time of:

$$t = 10\ injections * 25 \frac{min}{injection} + 2\ injections * 40 \frac{min}{injection} = \ 330\ min$$

The concentration of AcN in the mobile phase is 270 ml/l and 300 ml/l for nicotine and preservatives, and NRS, respectively. Both methods use a flow (*F*) of 2 ml/min, giving following:

$$F_{AcN,NIC-pres} = 2 \frac{ml}{min} * 270 \frac{ml\ AcN}{l\ mobile\ phase} = 0.54\ \frac{ml\ AcN}{min}$$

$$F_{AcN,NRS} = 2 \frac{ml}{min} * 300 \frac{ml\ AcN}{l\ mobile\ phase} = 0.6\ \frac{ml\ AcN}{min}$$

Combining the flow of AcN with the analysis times for each method gives:

$$V_{AcN,NIC-pres} = 0.54 \frac{ml\ AcN}{min} * 325\ min = 175.5\ ml\ AcN$$

$$V_{AcN,NRS} = 0.6 \frac{ml\ AcN}{min} * 330\ min = 198\ ml\ AcN$$

Leading to a total volume of 373.5 ml AcN for currently used methods.

For the **new method**, external calibration is used with 4 standard solutions. No duplicates are performed, leading to a total of 13 injections. The flow used is 0.8 ml/min. Since a gradient is used, the amount of organic solvent (B) changes with time, see table B1.

Table B1: Gradient of mobile phase for developed method. B represents organic solvent.

Time	% B	ml B
0	0.1	
1.2	3	0.014
2.2	10	0.028
6.5	20	0.17
7.5	95	0.30
8	0.1	0.19
10	0.1	0
Totally (ml/inj)		0.70

To clarify, the volume of organic solvent consumed between two times is calculated by using following:

$$V_{org} = \frac{\left(F * |t_2 - t_1| * \frac{|B_2 - B_1|}{100}\right)}{2}$$

Thus, since the analysis sequence include 13 injections, totally used organic solvent is:

$$V_{org} = 0.7 \ \frac{ml\ organic\ solvent}{injection} * 13\ injections = 9.15\ ml\ organic\ solvent$$

Comparing this volume with the present methods leads to approximately 40 times less consumption of organic solvent, see equation below.

$$\frac{373.5}{9.15} = 40.8$$

8.3 Appendix C

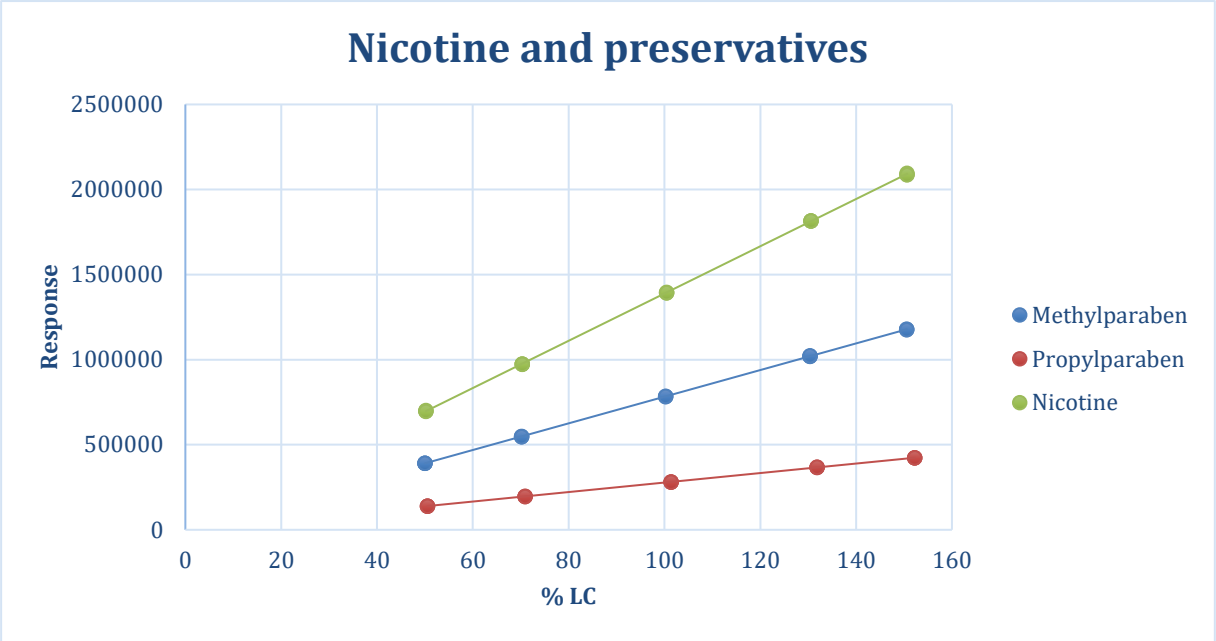

Figure C1: Response vs %LC obtained from the linearity presented for nicotine and preservatives.

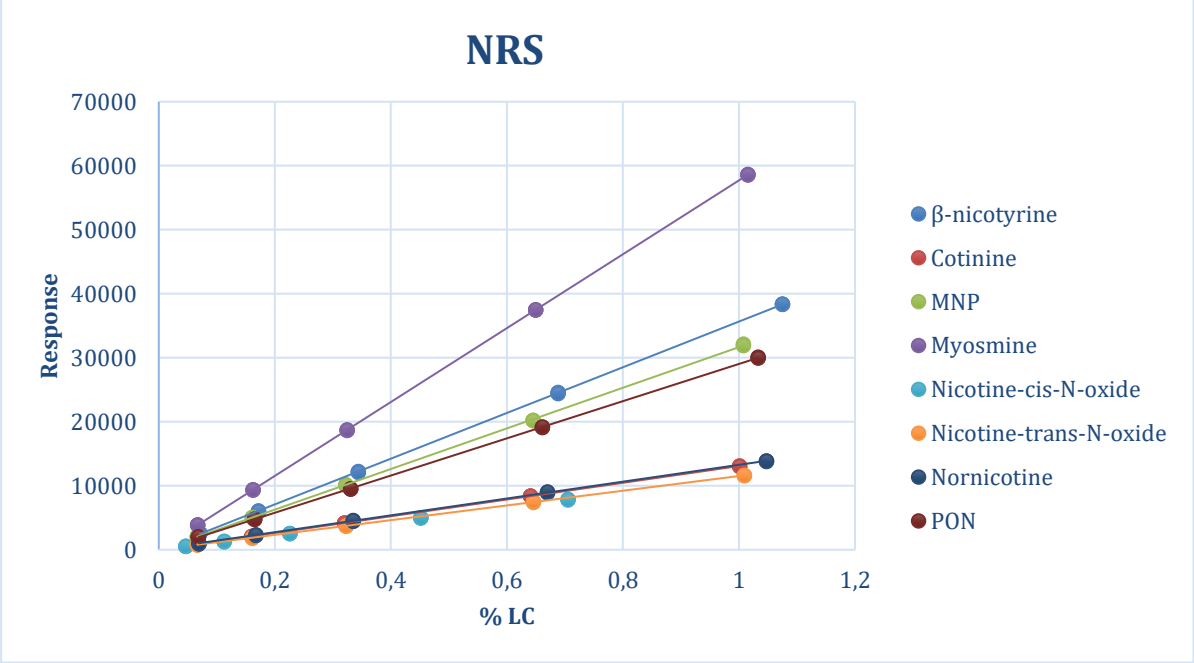

Figure C2: Response vs %LC obtained from the linearity presented for each NRS.