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Rapid, sensitive, and reliable quantitation of nicotine and its main metabolites cotinine and *trans*-3′-hydroxycotinine by LC-MS/MS: Method development and validation for human plasma

Nadja Mallock [a,b,*], Andrea Rabenstein [c], Peter Laux [a], Tobias Rüther [c], Christoph Hutzler [a], Maria Kristina Parr [b], Andreas Luch [a,b]

[a] German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany
[b] Institute of Pharmacy, Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, Germany
[c] Department of Psychiatry, Ludwig-Maximilians-Universität (LMU), Nussbaumstrasse 7, 80336 Munich, Germany

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ABSTRACT

New nicotine delivery products are gaining market share. For evaluation of their characteristics, toxicokinetic investigations are in current research focus. For reliable determination of blood plasma levels of nicotine and its main metabolites cotinine and *trans*-3′-hydroxycotinine, a quantitation method based on LC-ESI-MS/MS was developed and validated. Addition of isotope labeled internal standards prior to rapid sample preparation using protein precipitation with methanol was chosen for sample preparation. Different stationary phases were tested and phenyl-hexyl separation was found to be superior to HILIC, C18, and C8 stationary phases. Ion suppression effects caused by hydrophilic early eluting matrix were eliminated by the adjustment of an adequate retention utilizing a phenyl-hexyl separation stationary phase. Exchange of acetonitrile as organic mobile phase by methanol and elevation of pH value of aqueous mobile phase containing 5 mM NH_4Ac to 4.50 improved the chromatographic resolution. The limits of quantitation for nicotine, cotinine, and hydroxycotinine were 0.15, 0.30, and 0.40 ng/mL, respectively. Linearity was proven by matrix matched calibration for the whole working range from 0.50 ng/mL to 35.0 ng/mL for nicotine and from 6.00 to 420 ng/mL for cotinine and hydroxycotinine (Mandel's fitting test with $R^2 > 0.995$). Quality control samples at four different levels (0.50, 1.50, 17.5, 28.0 ng/mL for nicotine and 6.00, 18.0, 210, 336 ng/mL for cotinine and hydroxycotinine) in plasma were analyzed six times on three days. Mean accuracies ranged from 87.7% to 105.8% for nicotine, from 90.3% to 102.9% for cotinine, and from 99.9% to 109.9% for hydroxycotinine. Intra- and inter-day precisions (RSD %) were below 15% for all analytes (<20% for LLOQ). As proof of concept, the method was successfully applied to a real plasma sample from a cigarette smoking volunteer.

1. Introduction

Blood levels of nicotine after cigarette smoking are an important factor in monitoring of the development and maintenance of nicotine addiction [1–3]. Nicotine replacement therapy (NRT) in smoking cessation is based on the adjustment of a nicotine level in the body that sufficiently suppresses the urge to smoke [3]. Also new products like electronic cigarettes and heated tobacco products are discussed as replacements for combustible cigarettes and as cessation aids [4,5]. However, public health risks like uptake of cigarette smoking by non-smokers are discussed for these products [4–6]. When possible risks and chances of these new products are evaluated, nicotine delivery and toxicokinetics are important factors that need to be studied.

After inhalation, nicotine is rapidly absorbed in the small airways and reaches the brain after 10–20 s. It is widely distributed in the body and undergoes extensive metabolism [1–3]. The most important route of metabolism is mediated via cytochrome P450 (CYP) isoform 2A6 and results in the metabolites cotinine and *trans*-3′-hydroxycotinine (in the following only referred to as hydroxycotinine) as displayed in Fig. 1a [1–3]. The ratio of hydroxycotinine and cotinine is referred to as

* Corresponding author at: German Federal Institute for Risk Assessment (BfR), Department of Chemical and Product Safety, Max-Dohrn-Str. 8-10, 10589 Berlin, Germany.
E-mail address: Nadja.Mallock@bfr.bund.de (N. Mallock).

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"nicotine metabolic ratio" and is used as an important surrogate marker for CYP 2A6 activity and consequently the status of nicotine metabolism [7–10]. The kinetics of nicotine metabolism is considered to be an important factor for the success of NRT [7]. Studies have shown that slow metabolizers respond better to some types of NRT compared to normal metabolizers. This may be caused by higher nicotine blood levels [10].

Although several validated LC-MS/MS methods for separation of nicotine and metabolites were already published, they did not fit our needs entirely. For example, some groups analyzed nicotine and metabolites using stationary phases with hydrophilic-lipophilic interaction (HILIC) separation principles [11–16]. In all cases, sample preparation includes purification with solid phase extraction (SPE) or liquid–liquid extraction (LLE). Other separation principles like reversed-phase chromatography could be used in combination with a less extensive sample clean-up. Further, phenyl-hexyl based stationary phases combine reverse phase separation with additional retention mechanisms like π-π interactions between the stationary phase and the analytes [17–21]. If analytes contain π-electron systems, for example in aromatic rings, the retention can be enhanced by π-π interactions.

Reliable determination of nicotine and its metabolites cotinine and hydroxycotinine requires a suitable quantitation method that is selective, robust and reproducible. Further, a high sensitivity (LOQ < 0.5 ng/mL nicotine) and quick and easy sample preparation are required for large sample series. To meet all of these criteria, a method based on liquid chromatography coupled to tandem mass spectrometry with electrospray ionization (LC-ESI-MS/MS) applying protein precipitation was developed. The choice of the stationary phase, optimization of chromatography on the phenyl-hexyl stationary phase, the subsequent validation of optimized method, and proof-of-concept application of our developed method on real samples is presented herein.

2. Material and methods

2.1. Chemicals and reagents

All solvents and chemicals were of purity grade suitable for mass spectrometry. (-)-Nicotine (purity $\geq$ 99%), (±)-nicotine-(methyl-d_3) (isotopic purity $\geq$ 99%), solutions of (-)-cotinine (purity $\geq$ 99%), (±)-cotinine-(methyl-d_3) (isotopic purity $\geq$ 99%), *trans*-3′-hydroxycotinine (purity $\geq$ 98%; all 1.0 mg/mL in methanol), *trans*-3′-hydroxycotinine-d_3 (isotopic purity $\geq$ 99%; 100 μg/mL in methanol), and ammonium formate were purchased from Sigma Aldrich (Taufkirchen, Germany). Methanol, acetonitrile, formic acid, and fetal calf serum (FBS superior) were bought from Merck KGaA (Darmstadt, Germany) and ammonium acetate from Honeywell Fluka (Morris Plains, NJ, USA). Nitrogen gas was obtained from Linde (Pullach, Germany) with a purity of 99.999%. Ultrapure water was prepared with a Milli-Q Integral Water Purification System (Merck KGaA, Darmstadt, Germany).

2.2. Human plasma

Human plasma was obtained from healthy volunteers. Blank plasma was donated by non-smokers and tested with the herein described LC-ESI-MS/MS method for impurities prior to use. For proof of concept, venous blood was collected into EDTA monovettes (Sarstedt, Nümbrecht, Germany) from a routine cigarette smoker while smoking a combustible cigarette. The study was approved by the ethics committee of the LMU Munich and performed in accordance with the principles of the Declaration of Helsinki. Full blood was centrifuged for 10 min at 1,500g and 4 °C. To 990 μL sample plasma, 10 μL internal standard mix (see 2.3) was added. Samples were stored at −80 °C and shipped on dry ice.

2.3. Stock solutions, internal standard mix, matrix calibration, and quality control samples

Stock solutions of nicotine, cotinine, and hydroxycotinine were separately prepared in methanol and stored at −20 °C. They were used for the preparation of matrix calibration samples and quality control samples. Stock solutions of internal standards nicotine-d_3, cotinine-d_3, and hydroxycotinine-d_3 were prepared in methanol and stored at −20 °C. A mix was prepared in acetonitrile with a concentration of 500 ng/mL for each internal standard. A reference standard mix (matrix-mix) with 50.0 ng/mL nicotine, 600 ng/mL cotinine, and 600 ng/mL hydroxycotinine was prepared in human blank plasma. Matrix calibration and quality control samples were prepared by spiking different volumes of standard mix into human blank plasma. To 990 μL spiked plasma, 10 μL internal standard mix was added, resulting in a concentration of 5.00 ng/mL per internal standard. Samples were stored at −80 °C. Concentrations of matrix calibration samples and quality control samples are summarized in Table 1.

2.4. Sample preparation

Frozen samples were gently thawed on ice. To 50 μL of plasma, 100 μL of ice-cold methanol was added for protein precipitation. After thorough mixing for 30 s on a vortex shaker (7–2020, neoLab Migge GmbH, Heidelberg, Germany), samples were centrifuged at 4 °C and 14,000 g for 15 min (Centrifuge 5427 R, Eppendorf, Wesseling-Berzdorf,

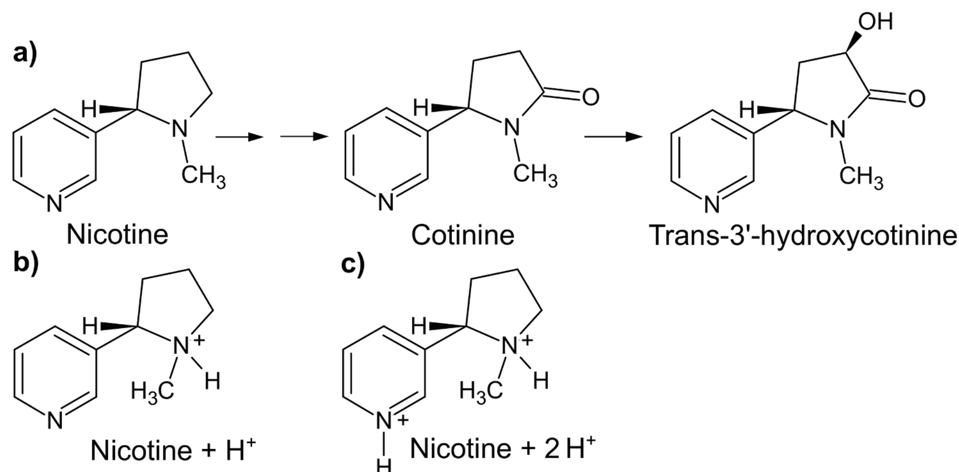

Fig. 1. **a)** Metabolism of nicotine to its main metabolites cotinine and *trans*-3′-hydroxycotinine. **b)** Protonated nicotine (pyrrolidine nitrogen atom protonated) as dominant form at neutral pH. **c)** Doubly protonated nicotine species (pyridine nitrogen atom protonated as well) as dominated form below pH 3.10.

Table 1
Concentrations of analytes in matrix calibration and quality control samples.

Standard	Nicotine (ng/mL)	Cotinine (ng/mL)	Hydroxycotinine (ng/mL)
Matrix calibration samples			
K1 (LLOQ)	0.50	6.00	6.00
K2	2.50	30.0	30.0
K3	5.00	60.0	60.0
K4	10.0	120	120
K5	15.0	180	180
K6	20.0	240	240
K7	25.0	300	300
K8	30.0	360	360
K9 (ULOQ)	35.0	420	420
Quality control samples			
LLOQ	0.50	6.00	6.00
Low QC (3x LLOQ)	1.50	18.0	18.0
Mid QC (50% ULOQ)	17.5	210	210
High QC (80% ULOQ)	28.0	336	336

Germany). Supernatant was diluted 1:1 with aqueous eluent A to reduce the methanol content for a better separation. The resulting sample solution was used directly for LC-ESI-MS/MS analysis.

2.5. *LC-ESI-MS/MS*

Analysis was performed with a liquid chromatography system consisting of pumps (LC-20AD), degasser (DGU-20As), auto sampler (SIL-20AC HT), column oven (CTO-20AC), and communications bus module (CBM-20A; all from Prominence series, Shimadzu, Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API4000QTrap, AB Sciex, Framingham, MA, USA) equipped with an electrospray ion source (ESI) operated with Analyst 1.7 software (AB Sciex, Framingham, MA, USA). After injection of 25 µL of final sample solution, separation was achieved on a Luna Phenyl-Hexyl Column (150 mm length, 4.60 mm internal diameter, 3 µm particle size, 100 Å pore size; Phenomenex, Torrance, CA, USA) equipped with an according guard column (Phenomenex, Torrance, CA, USA) at 45 °C. To prepare eluent A, formic acid was added to 5 mM ammonium acetate in ultrapure water to adjust pH 4.50 ± 0.02 (controlled with 765 Calimatic pH meter; Knick, Berlin, Germany). Methanol was used as eluent B. At a total flow of 1 mL/min, the following gradient was used: Start at 10% B, followed by an increase for 1 min to 30% B with a hold for 1 further min, followed by another increase to 95% B for 2 min and a hold for 2 min, followed by a decrease to 10% for 0.2 min and a hold for 2.8 min. Conditions at the ESI-source were as followed: ion spray voltage, 3800 V; ion source temperature, 650 °C; curtain gas, nitrogen with 10 psi; ion source gas 1, nitrogen with 80 psi; ion source gas 2, nitrogen with 85 psi. Declustering potential was set to 47 V and entrance potential was 7 V. Mass selective detection was performed with multiple reaction monitoring (MRM) mode in positive mode with two transitions per analyte and a detection window of 120 s and a cycle time of 1 s. MRM parameters are summarized in Table 2. In the final method, scheduled multiple reaction monitoring was applied to all transitions. Product ion scan mass spectra of all analytes and internal standards recorded with a collision energy of 52 V are displayed in Figure S.4 (Supplementary Material). Data was analyzed with Software ScieX OS (Version 1.4.0.18067, AB Sciex, Framingham, MA, USA) using the same “Autopeak” integration parameters for all measurements.

2.6. *Stationary phase selection*

Separation with C18 (EC Nucleosil 100–5 HD C18 column, 150 mm length, 4.60 mm internal diameter, 5 µm particle size, 100 Å pore size; Macherey-Nagel, Düren, Germany) and C8 (EC Nucleosil 120–3 C8 column, 150 mm length, 4.60 mm internal diameter, 3 µm particle size, 120 Å pore size; Macherey-Nagel, Düren, Germany) stationary phases was performed as described under 2.5. In both cases, two mobile phase compositions were tested: 5.00 mM ammonium acetate, 0.1% formic acid in ultra-pure water (eluent A) and the same modifiers in acetonitrile or methanol (eluent B), and also eluent A (5 mM ammonium acetate, formic acid until pH 4.50 in ultra-pure water) and eluent B (methanol) as described under 2.5. Prior to injection, a mix of all standards in methanol (60.0 ng/mL) was diluted 1:2 with the according aqueous eluent A to have the same amount of methanol as in the matrix samples. Separation of matrix samples on C8 and C18 stationary phases has not been tested due to an insufficient separation and bad peak shapes even for matrix-free standards. For this experiment, data acquisition in MRM-mode was not scheduled, but recorded with a fixed dwell time of 70 ms for each transition. Additionally, a HILIC stationary phase (Luna HILIC column, 150 mm length, 3.00 mm internal diameter, 3 µm particle size, 200 Å pore size; with HILIC guard column; Phenomenex, Torrance, CA, USA) was used with an isocratic flow of 5 mM ammonium formate in 95% acetonitrile and 5% ultra-pure water at 0.40 mL/min and 40 °C. Sample preparation prior to HILIC separation was performed as described under 2.4 with the exceptions that proteins were precipitated with 150 µL acetonitrile and that the supernatant was not diluted after centrifugation. At this early stage of method development, fetal calf serum was used as a surrogate matrix, as it is more accessible than nicotine-free human plasma.

Table 2
Parameters for MRM-transitions of quantifiers and qualifiers of analytes and internal standards.

	Q1 mass (Da)	Q3 mass (Da)	Retention time (min)	Collision energy (volts)	Collision exit potential (volts)
Nicotine					
Quantifier	163.2	130.0	3.20	29	6
Qualifier	163.2	132.1	3.20	21	24
Nicotine-d_3					
Quantifier	166.3	132.0	3.20	23	6
Qualifier	166.3	130.0	3.20	45	6
Cotinine					
Quantifier	177.2	98.0	5.20	40	18
Qualifier	177.2	80.0	5.20	25	14
Cotinine-d_3					
Quantifier	180.2	80.0	5.20	35	14
Qualifier	180.2	101.0	5.20	31	18
Hydroxycotinine					
Quantifier	193.1	80.0	4.40	43	14
Qualifier	193.1	134.1	4.40	27	24
Hydroxycotinine-d_3					
Quantifier	196.2	80.0	4.40	41	14
Qualifier	196.2	134.1	4.40	27	24

2.7. *Testing of different mobile phases*

Eluent A containing 5 mM ammonium acetate in ultra-pure water was adjusted to different pH values by addition of formic acid: pH 2.86 (addition of 0.1% formic acid), pH 3.00, pH 3.50, pH 4.00, pH 4.20, pH 4.30, pH 4.40, pH 4.44, pH 4.50, pH 4.60. Eluent B was prepared with 5 mM ammonium acetate and 0.1% formic acid (and 5% water for acetonitrile) or without modifiers using methanol or acetonitrile. Prior to injection, a mix of all standards in acetonitrile or methanol (60.0 ng/mL) was diluted 1:2 with the according aqueous eluent A to have the same amount of methanol as in the matrix samples. For this experiment, data acquisition in MRM-mode was not scheduled, but recorded with a fixed dwell time of 70 ms for each transition. The Henderson-Hasselbalch equation ($pH = pK_a - \log (C_{acid}/C_{base})$) was used to calculate the proportion of charged analyte molecules [22].

2.8. *Characterization of ion suppression*

Blank solution (methanol diluted with twofold eluent A) or human blank plasma, prepared as described under 2.4, were analyzed as

described under 2.5. Analyte solution (500 ng/mL in methanol) was infused continuously post-column at a constant flow rate of 20 µL/min using a syringe pump (11 Plus, Harvard Apparatus, March-Hugstetten, Germany) equipped with a 1 mL luer lock syringe (Gastight, Hamilton, Gräfelfing, Germany), while running the analysis of a blank matrix sample. The intensity of the MRM signals for all analytes was monitored over time to characterize ion suppression regions in the chromatogram.

2.9. Characterization of matrix effects

Matrix effects were determined for plasma samples from six different anonymous donors based on EMA Guideline on bioanalytical method validation [23]. Concentrations of Low QC (1.50 ng/mL nicotine, 18.0 ng/mL cotinine, hydroxycotinine) and High QC (28.0 ng/mL nicotine, 336 ng/mL cotinine, hydroxycotinine) were spiked together with internal standard mix (5.00 ng/mL each internal standard in the spiked sample) in the different plasma samples. The same analyte matrix-mix was used as stock to prepare the samples (Low QC: 960 µL plasma sample + 10 µL internal standard mix + 30 µL analyte matrix-mix; High QC: 430 µL plasma sample + 10 µL internal standard mix + 560 µL internal standard mix), because there was not enough nicotine-free plasma available to prepare different analyte matrix-mixes for each sample. Matrix samples were analyzed against matrix-free control samples with the same concentrations. Matrix-free control samples were prepared in methanol and diluted with eluent A to the same ratio of methanol to aqueous part (1:2, v/v) as in the final sample solution. To reach the same concentrations in the final sample, concentrations in methanol prior to dilution were 0.75 ng/mL nicotine, 9.00 ng/mL cotinine and hydroxycotinine, 2.50 ng/mL internal standards for Low QC and 14.0 ng/mL nicotine, 168 ng/mL cotinine and hydroxycotinine, 2.50 ng/mL internal standards for High QC. Internal standard-normalized matrix factors were calculated as described in the Supplementary Material.

2.10. Method validation

Definitions, methods, and criteria for validation were based on international guidelines [23,24]. The criteria that were defined for a successful validation are summarized in Table 3. While most validation experiments were performed according to the current bioanalytical guideline published by EMA [23], additional experiments were performed based on relevant in-house criteria and JRC guideline on methods used in controls of food contact materials [24]. For accuracy and precision, the matrix matched calibration and quality control samples were freshly spiked in pooled matrix at three different days. Matrix calibration samples were prepared (as described under 2.4) and analyzed twice. Quality control samples were prepared and analyzed six times. One of the six resulting sample solutions per quality control level was injected six times in total to assess precision of the instrument. Accuracy was calculated by dividing the found concentrations by the nominal concentrations. Precision was calculated as relative standard deviation. Concentrations resulting in a signal to noise ratio of 3 was defined as limit of detection (LOD) and signal to noise ratio of 10 as limit of quantitation (LOQ). To test for selectivity, six blank plasma samples from different donors were analyzed and checked for interferences for all MRM transitions at the relevant retention times. Assessment of intra-laboratory repeatability was performed additionally to bioanalytical guidelines using different analyte concentrations in the quality control samples (0.75, 12.5, 22.5, 32.5 ng/mL nicotine, 9.00, 150, 270, 390 ng/mL cotinine and hydroxycotinine): Each quality control sample was prepared six times for measurement as described in Section 2.4 to assess precision of sample preparation. One of these quality control samples for each level was injected six times to assess precision of the instrument. The procedure was repeated twice, once by the same person to determine inter-day precision and again by another operator to assess within-laboratory reproducibility.

Table 3
Validation criteria.

Parameter	Criteria
Validation criteria according to bioanalytical guidelines [23]	
Selectivity	No interferences in 6 different matrix samples (response < 20% response of LLOQ for analytes, < 5% for internal standards)
Linearity	Accuracy of at least 75% of calibration samples is 85 – 115% (80 – 120% for LLOQ) with at least 6 calibration points
Accuracy	85 – 115% (80 – 120% for LLOQ)
Precision	≤ 15% (≤20% for LLOQ)
Stability	85 – 115% of nominal value Benchtop: for 5 h on ice and at room temperature Storage: −80 °C for 3 months Freeze and thaw: for at least 3 cycles In autosampler: for 24 h at 15 °C
Matrix factor	CV of ISTD-normalized matrix factors from 6 different matrices ≤ 15%
Additional validation criteria according to other guidelines [24] and in-house criteria	
Selectivity	Stability of retention time (±5%); Stable ratio of quantifier and qualifier MRM (±20% deviation);
Linearity	Linear according to Mandel's fitting test and correlation coefficient $R^2 > 0.995$ with at least 6 calibration points covering the whole working range, weighting 1/x
Within-laboratory reproducibility	Difference between accuracies by different operators ≤ 20%

2.11. Stability under benchtop, freeze and thaw, and autosampler conditions

The stability of Low QC (1.50 ng/mL nicotine, 18.0 ng/mL cotinine, hydroxycotinine) and High QC (28.0 ng/mL nicotine, 336 ng/mL cotinine, hydroxycotinine) samples in matrix under defined conditions was determined. For the determination of the benchtop stability, matrix quality control samples were left at room temperature or on ice for up to 5 h. Samples were analyzed in triplicate after 0, 30, 60, 90, 120, 180, 240, and 300 min. Further, stability over 3 freeze and thaw cycles was assessed. Matrix quality control samples were analyzed in triplicate directly after they have been spiked and at three additional days. In between experiments, samples were kept at −80 °C for at least 12 h. They were completely thawed, analyzed in triplicate against a freshly prepared matrix calibration and refrozen at –80 °C. Stability under autosampler conditions was assessed with a triplicate preparation of matrix quality control samples. The resulting samples were divided into 2 vials with 100 µL each. The first set of samples was injected at the beginning of a sequence. The second set was injected at the end of the sequence after 24 h. The closed vials were kept in the autosampler at 15 °C during the 24 h time period.

2.12. Storage stability of frozen samples

The analytes were spiked separately into human plasma containing 5.00 ng/mL internal standard mix. Aliquots were stored at −20 and −80 °C. At days 0, 21, 35, 49, 63, 76, and 119, an aliquot was prepared as described under 2.4 and analyzed. Remaining supernatants were stored at −80 °C until all samples were analyzed again in one run. Recovery was calculated by dividing the measured concentrations after storage by concentrations at day 0 and multiplied with 100%.

3. Results and discussion

3.1. Selection of stationary phase

As a first step, two different separation principles were tested for our analytes: HILIC and reversed-phase separation. Resulting chromatograms are shown in Fig. 2. Using HILIC, injection of matrix-free analytes

in acetonitrile resulted in good separation and acceptable peak-shapes (Fig. 2a). However, when matrix samples were injected, peak shapes, especially of hydroxycotinine, got worse (Fig. 2b). It can be concluded that although HILIC chromatography is well-suited for our analytes, sample preparation protocols that do not remove hydrophilic matrix compounds can lead to significant matrix effects [25]. Since we aimed for a quick and easy sample preparation using only protein precipitation with solvents, the amount of plasma constituents in our samples is supposed to be problematic in combination with HILIC chromatography. Fewer problems are expected with reverse phase chromatography. Thus, a C18 stationary phase has been tested with acetonitrile (Fig. 2c) and with methanol as organic phases. In combination with methanol, chromatograms derived from two exemplary aqueous mobile phases are shown for pH 2.86 (Fig. 2d) and pH 4.50 (Fig. 2e). Separation and peak shapes of cotinine and hydroxycotinine improved when exchanging acetonitrile with methanol and further with increasing pH value. However, the broad peak shape for nicotine improved only slightly from exchanging acetonitrile with methanol, but worsened significantly with the increase of pH value. The same test on a C8 stationary phase (Fig. 2f-h) resulted in a similar observation. Separation of cotinine and hydroxycotinine was acceptable especially with the combination of methanol and pH 4.50. However, the more hydrophobic analyte nicotine did not elute as a defined peak with any of the tested mobile phases. The elution power of acetonitrile and methanol was not sufficient to elute nicotine as a sharp peak from both tested reversed-phase materials. It should be noted that mobile phase gradients were not optimized for C18 and C8 stationary phases. However, alteration of mobile phase gradient was not expected to affect chromatography of nicotine to a satisfactory extent.

Therefore, a phenyl-hexyl stationary phase that combines reverse phase separation with other retention mechanisms like π-π interactions between analytes and stationary phase was selected to improve chromatographic resolution and separation [17–21]. Since optimization of mobile phase plays an important role for separation of our analytes on a phenyl-hexyl column, mobile phase selection is presented under 3.2.

3.2. Selection of mobile phase

Two aspects of the mobile phase have been optimized to achieve good peak shapes especially for the main analyte nicotine: the organic solvent and the pH value. Firstly, acetonitrile in the mobile phase can weaken the influence of π-π interactions on retention [19,21,26,27]. Secondly, nicotine contains two basic nitrogen atoms that may get protonated, resulting in one or two positive charges. The nitrogen atom in the pyrrolidine moiety has basic properties with a pk_a of 8.01 [28]. The second nitrogen atom, located in the aromatic pyridine moiety, has a pk_a of 3.10 and can be protonated under acidic conditions (Fig. 1c) [28]. A positive charge of the pyridine moiety is unfavorable due to negative influence on π-π interactions. Further, to achieve a good peak shape in the chromatograms for the most important analyte nicotine, all nicotine molecules should carry the same charge of + 1. A mixture of differently charged nicotine molecules during chromatography is supposed to cause peak broadening or even double peaks. Thus, the proportion of nicotine molecules with charges at the two basic moieties was calculated using the Henderson-Hasselbalch equation [22]. The results are reported in Table 4. The pyrrolidine moiety is positively charged at all tested pH values. However, protonation of the pyridine ring was below 5% at pH 4.40, leading to acceptable peak shape of nicotine in the chromatogram. To confirm this prediction, actual peak shapes at the different pH values have been compared. Resulting chromatograms are shown in Figs. 3 and 4. For cotinine and hydroxycotinine, the pK_a of the pyridine moiety is expected to be similar to the one for nicotine. However, the introduction of an electronegative carbonyl group into the pyrrolidine moiety leads to a decrease in electron density and a reduction of the basic properties. Thus, the second nitrogen atom is not expected to be protonated at tested pH values.

Further, two different solvents were tested as organic eluent B: acetonitrile and methanol. Results for acetonitrile are shown in Fig. 3: At first, eluent A and B both contained the same modifiers, i.e., 5 mM ammonium acetate and 0.1% formic acid (pH = 2.86 in eluent A, Fig. 3a). Peak splitting for cotinine and peak broadening for nicotine was observed. Then, the pH of the aqueous eluent A was altered, and acetonitrile was used without modifiers (Fig. 3b-d). While cotinine eluted as a single peak, peak splitting was now observed for hydroxycotinine. Chromatography of nicotine did not improve and peak splitting was observed at pH 4.00 (Fig. 3c).

Fig. 4 displays the results obtained using methanol as organic solvent in eluent B. At first, eluent A and B contained the same modifiers (5 mM ammonium acetate and 0.1% formic acid, Fig. 4a). Two peaks for nicotine both with poor retention were observed. Then, pH value of the aqueous eluent A was adjusted and additive-free methanol was used as eluent B (Fig. 4b-i). With increasing pH, peak splitting of nicotine turned into fronting of a single peak at pH 4.00 (Fig. 4c) and 4.20 (Fig. 4d). A further increase resulted in better peak shape and improved retention for nicotine. Peak shape and retention and consequently intensity of cotinine and hydroxycotinine improved as well comparing chromatograms at pH 2.86 (Fig. 4a) and pH 4.50 (Fig. 4h). Chromatographic parameters such as retention time, peak height, full width at half maximum, and tailing factor at different tested pH values with methanol as eluent B are summarized in Table 5. While high values are favorable for the parameters retention time and peak height, full width at half maximum and tailing factors should be low. Chromatographic parameters and especially full width at half maximum were acceptable for all three analytes when determined at pH 4.50 and were sufficiently robust against pH changes. This confirmed that the suitability of the priorly calculated pH value of 4.50 for the aqueous mobile phase.

In addition to the hydrophobic interactions of regular reverse phase columns, phenyl-hexyl columns can achieve additional retention of compounds via π-π interactions. Acetonitrile weakened π-π interactions between analytes and stationary phase. Without the additional binding mechanism, chromatography of cotinine and hydroxycotinine was largely influenced by pH (Fig. 3). The elution order of the three analytes varied depending on the pH value. The use of methanol in contrast resulted in better retention and a different and stable elution order. These observations are in line with existing literature [19,21,27]. Consequently, it can be concluded that π-π interaction as additional retention mechanism improves the chromatography of nicotine, cotinine, and hydroxycotinine. When reversed-phase liquid chromatography is favored, a stationary phase with this additional retention mechanism should be considered and used with methanol and an appropriate pH value. For the final method, a pH value of 4.50 for eluent A (Fig. 4h) was selected. As presented in Table 5, differences in chromatography at pH 4.44 (Fig. 4g) and 4.60 (Fig. 4i) were found to be minor and the quality of the chromatographic separation seems to be robust against small variations in pH value of eluent A. In conclusion, the pH value of mobile phase A is considered as critical control parameter and the use of methanol as organic solvent was found superior compared to acetonitrile.

3.3. Characterization of ion suppression by co-eluting matrix and influence on matrix effects caused by different plasma donors

The aim of our method development was to combine quick sample preparation with high sensitivity, especially for nicotine. Protein precipitation is a quick and very easy sample preparation method, but hardly removes all possible kinds of matrix constituents that can lead to suppression of ionization in certain regions of the chromatogram. When electrospray ionization is used, co-eluting matrix can hamper the ionization of the analytes and result in reduced sensitivity [29–33]. These matrix effects are not limited to the solvent front and may occur due to co-elution of matrix constituents at any time during chromatography [29,31]. To achieve best sensitivity and reproducibility of quantitation,

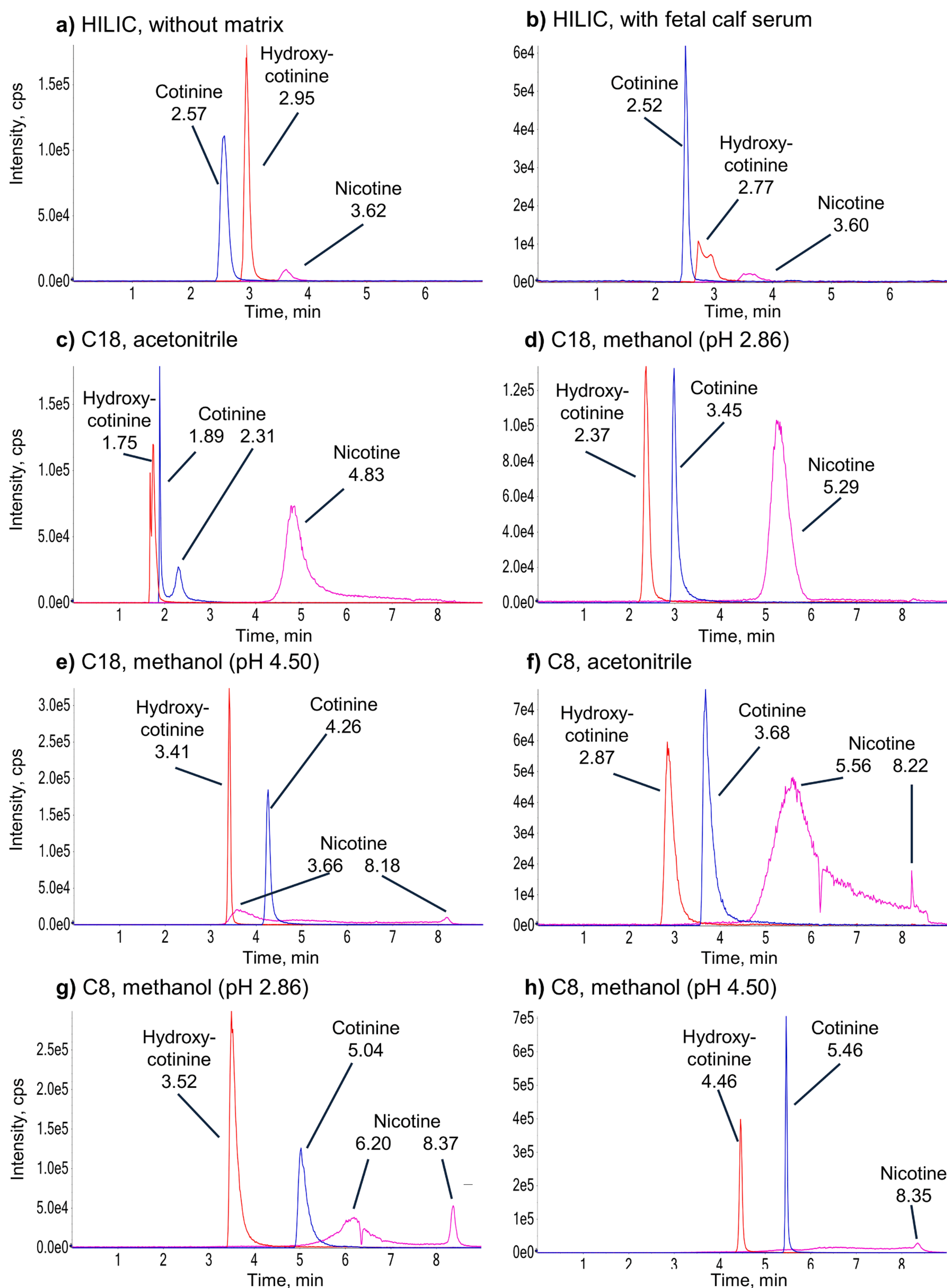

Fig. 2. Chromatograms resulting from different stationary phases: HILIC separation of analytes **a)** in acetonitrile (5.00 ng/mL nicotine, 120 ng/mL cotinine and hydroxycotinine) and **b)** in fetal calf serum after protein precipitation. Separation of analytes without matrix on **c)**, **d)**, and **e)** a C18 stationary phase and **f)**, **g)**, and **h)** a C8 stationary phase. Mobile phases were for **c)** and **f)** 5 mM ammonium acetate, 0.1% formic acid in ultra-pure water (pH 2.86) and the same modifiers in acetonitrile, for **d)** and **g)** 5 mM ammonium acetate, 0.1% formic acid in ultra-pure water (pH 2.86) and the same modifiers in methanol, and for **e)** and **h)** ultra-pure water with 5 mM ammonium acetate and addition of formic acid until pH 4.50 was reached and methanol without modifiers.

Table 4
Proportion of nicotine molecules with positive charge at pyrrolidine and/or at pyridine moiety at different mobile phase pH values.

	Pyrrolidine moiety of nicotine	Pyridine moiety of nicotine
pK_a from [28]	8.01	3.1
pH	% charged	% charged
2.86	100%	63%
3.50	100%	28%
4.00	100%	11%
4.20	100%	7%
4.30	100%	6%
4.40	100%	5%
4.44	100%	4%
4.50	100%	4%
4.60	100%	3%

analytes should not elute at retention times where ion suppression occurs. To test for ion suppression, analyte solution was infused post-column as described under 2.7, based on a procedure suggested by Bonfiglio *et al.* [30]. Intensities of nicotine, cotinine, and hydroxycotinine quantifiers are visualized in Fig. 5. In comparison to matrix-free blank (Fig. 5a), strong ion suppression between 1.5 min and 2 min is observed when matrix is injected (Fig. 5b). The increase of intensity after 5.5 min is due to the high methanol content of 95% in the eluate at that time. High volatility and low surface tension of methanol and the low content of salts like ammonium acetate can improve droplet formation and thus ionization [31]. Since the analytes elute between 3.2 min and 5.2 min, effects of matrix are minor and can be compensated with isotope-labeled internal standards. Although the injected matrix is still complex after the quick sample preparation step, the separation method accomplishes adequate retention of analytes to avoid negative effects due to ion suppression.

To determine differences in matrix effects caused by different plasma donors, internal standard-normalized matrix factors were calculated with nicotine-free venous plasma from six different human donors at two concentrations and are presented in the Supplementary Material (Table S.5). Coefficients of variance (CV) between the six different matrix samples were analyzed per analyte and concentration. CV ranged from 1.3% to 4.9% and were well below the requirement of $\leq$ 15%.

3.4. Method validation

Calibration curves from day 1 are provided in Figure S.3 for all analytes (Supplementary Material). Results for accuracy and precision tested at 3 days are summarized in Table 6. All criteria from Table 3 that were set prior to validation were fulfilled. Linearity was proven with Mandel's fitting test over the used concentration range with correlation coefficients of the linear regressions (weighted 1/x) higher than 0.999 on day 1. >75% of matrix calibration samples were within $\pm$ 15% of the nominal value ($\pm$20% for LLOQ) as summarized in the Supplementary Material (Table S.1). Mean accuracies of quality control samples ranged from 87.7% to 109.9%. The precisions of sample preparation and the instrument were below 15% (below 20% for LLOQ) within one day and between three days. As presented in the Supplementary Material (Figure S.1 and Figure S.2), analysis of 6 different blank matrix samples showed no interferences with analytes or internal standards. Mean and standard deviation of retention times were 3.29 $\pm$ 0.05 min for nicotine, 3.27 $\pm$ 0.05 min for nicotine-d_3, 5.21 $\pm$ 0.01 min for cotinine, 5.20 $\pm$ 0.01 min for cotinine-d_3, 4.36 $\pm$ 0.02 min for hydroxycotinine, and 4.33 $\pm$ 0.02 min for hydroxycotinine-d_3, and were the same for quantifier and qualifier MRM. The maximum deviation of $\pm$ 5% was not exceeded. Ratios of quantifier and qualifier MRM were found to be within the tolerance of $\pm$ 20% for all analytes. Mean and standard deviations of ion ratios were 96.6 $\pm$ 7.2% for nicotine, 41.8 $\pm$ 6.1% for nicotine-d_3, 352.7 $\pm$ 3.4% for cotinine, 34.2 $\pm$ 2.4% for cotinine-d_3, 53.4 $\pm$ 6.6% for hydroxycotinine, and 50.3 $\pm$ 4.2% for hydroxycotinine-d_3. Suitable MRM-transitions for quantifiers and qualifiers were selected during method development and optimized individually for the three analytes

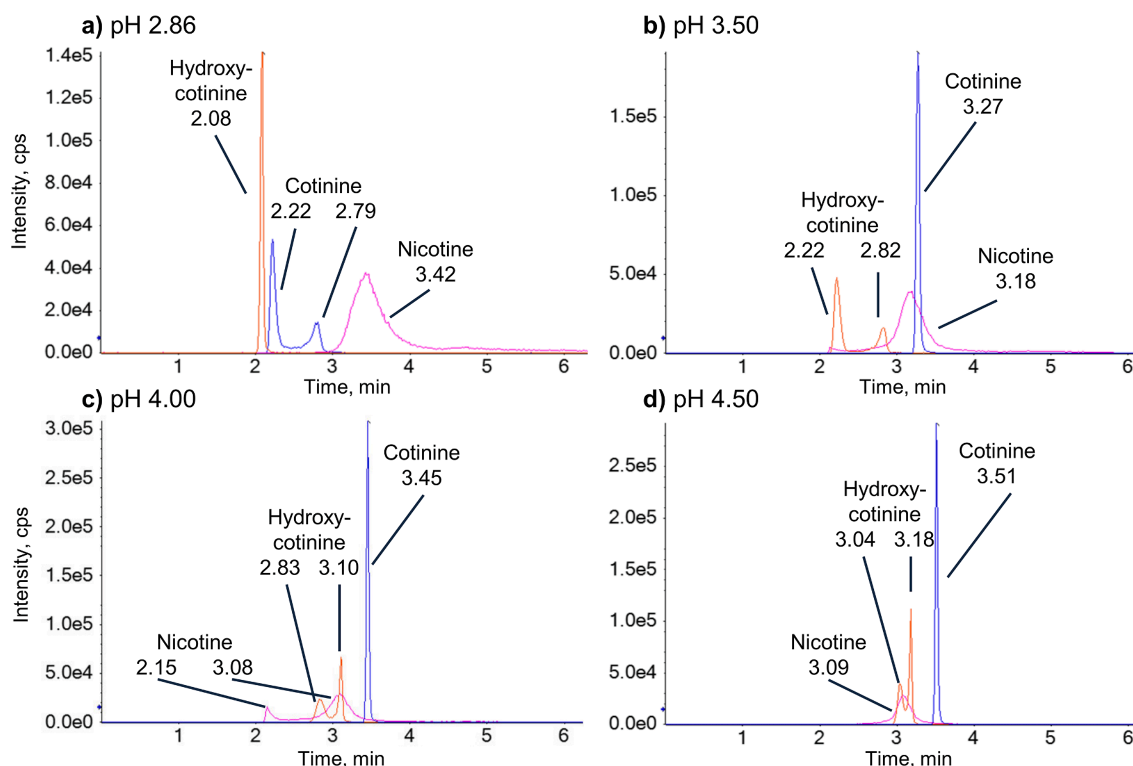

Fig. 3. Chromatograms resulting from different mobile phases: acetonitrile as eluent B containing **a)** 5 mM ammonium acetate, 5% water, and 0.1% formic acid, or **b-d)** without modifiers. Eluent A consisted of 5 mM ammonium acetate in water with **a)** 0.1% formic acid (pH 2.86) or addition of formic acid until **b)** pH 3.50, **c)** pH 4.00, or **d)** pH 4.50 was reached.

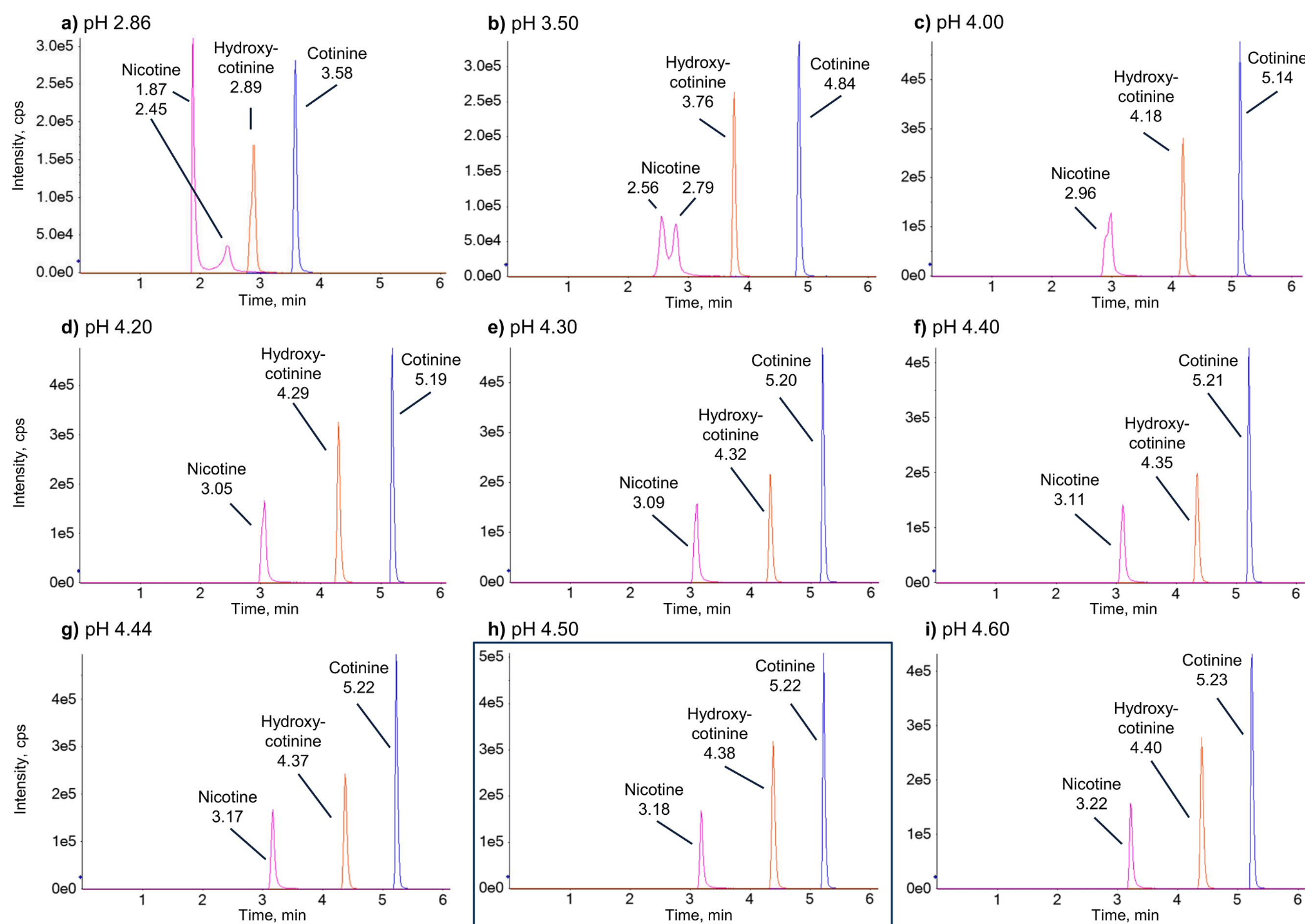

Fig. 4. Chromatograms resulting from different mobile phases: methanol as eluent B containing **a)** 5 mM ammonium acetate and 0.1% formic acid, or **b-i)** without modifiers. Eluent A consisted of 5 mM ammonium acetate in water with **a)** 0.1% formic acid (pH 2.86) or addition of formic acid until **b)** pH 3.50, **c)** pH 4.00, **d)** pH 4.20, **e)** pH 4.30, **f)** pH 4.40, **g)** pH 4.44, **h)** pH 4.50, or **i)** pH 4.60 was reached.

Table 5
Retention time (RT, in min), peak height (in cps), full width at half maximum (FWHM, in min), and tailing factor (Tf) for all analytes at different pH values of eluent A with methanol as eluent B.

pH	2.86	3.5	4	4.2	4.3	4.4	4.44	4.5	4.6
Nicotine									
RT	1.9	2.7	3.0	3.1	3.1	3.1	3.2	3.2	3.2
Height	3.1×10^5	8.6×10^4	1.2×10^5	1.5×10^5	1.5×10^5	1.3×10^5	1.5×10^5	1.4×10^5	1.3×10^5
FWHM	0.04	0.32	0.08	0.09	0.08	0.08	0.07	0.06	0.06
Tf	11.51	2.64	0.86	1.08	1.24	1.30	1.32	1.52	1.64
Cotinine									
RT	3.6	4.8	5.1	5.2	5.2	5.2	5.2	5.2	5.2
Height	2.8×10^5	3.4×10^5	4.8×10^5	4.8×10^5	4.7×10^5	4.3×10^5	4.9×10^5	5.1×10^5	4.3×10^5
FWHM	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04
Tf	1.14	1.22	1.78	1.32	1.78	1.64	1.50	1.61	1.24
Hydroxycotinine									
RT	2.9	3.8	4.2	4.3	4.3	4.4	4.4	4.4	4.4
Height	1.7×10^5	2.6×10^5	2.8×10^5	3.2×10^5	2.2×10^5	2.0×10^5	2.4×10^5	3.2×10^5	2.8×10^5
FWHM	0.07	0.05	0.05	0.05	0.06	0.06	0.06	0.06	0.06
Tf	0.98	1.14	1.19	1.39	1.37	1.19	1.44	1.31	1.35

with regard to signal-to-noise ratios and linearity of the working range. For cotinine, the MRM-transition with the highest intensity was used as qualifier instead of quantifier to obtain the best possible linear fit over the whole working range. Standard deviations of ion rations were highest when low concentrations of nicotine and high concentrations of cotinine were analyzed since they were close to lower or upper end of the linear range. Results for intra-laboratory repeatability that was assessed additional to bioanalytical guidelines using other concentrations of quality control samples (0.75, 12.5, 22.5, 32.5 ng/mL nicotine, 9.00, 150, 270, 390 ng/mL cotinine and hydroxycotinine) are presented in the Supplementary Material (Table S.6). Deviation of accuracies between operators was below 20%. The method was repeatable and reproducible within the laboratory. Estimated LOD and LOQ are shown in Table 7. The required sensitivity has been achieved.

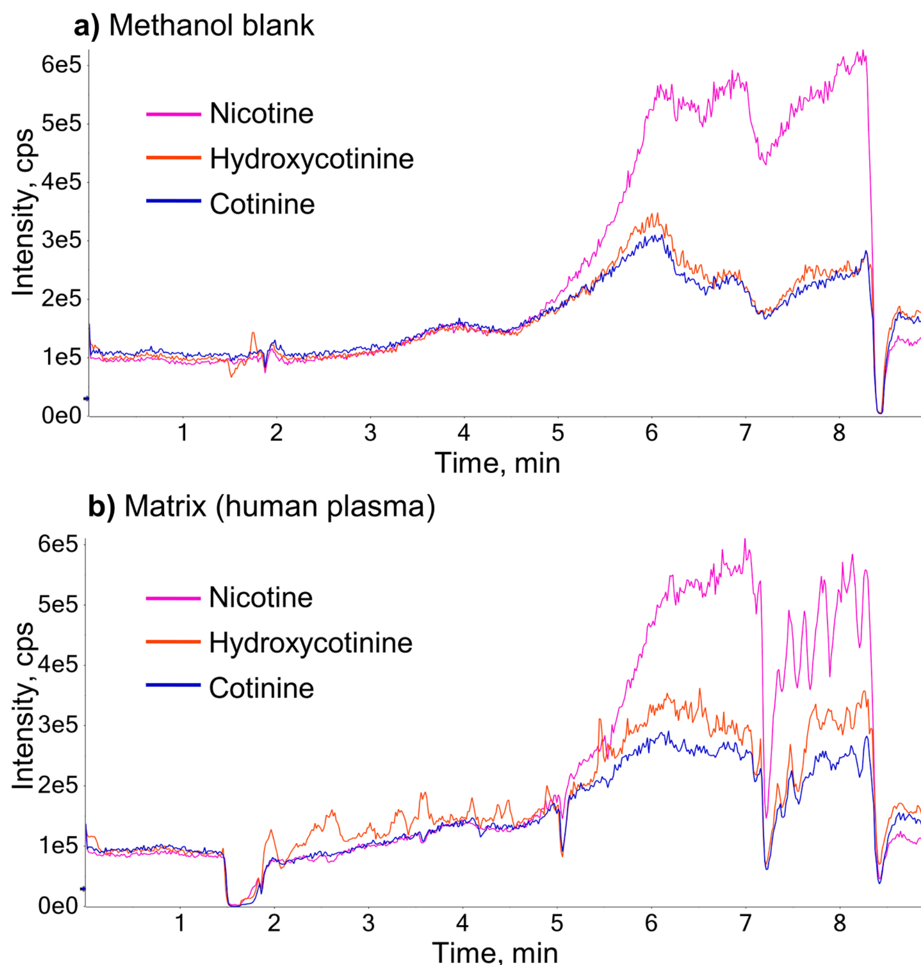

Fig. 5. Influence of eluting **a)** blank or **b)** matrix on intensities of post-column infused analytes.

Table 6
Overview of validation results: Accuracy and precision.

Concentration ng/mL	Day 1 Accuracy (%)	Precision (%)	Precision of injection (%)	Day 2 Accuracy (%)	Precision (%)	Day 3 Accuracy (%)	Precision (%)	Intra-day precision (%)
Nicotine								
0.50 (LLOQ)	105.8	9.3	9.6	87.7	7.7	96.0	16.3	13.6
1.50	98.5	6.0	5.5	89.8	5.0	97.4	6.7	7.0
17.5	97.1	1.2	1.4	100.8	6.6	95.1	4.0	4.9
28.0	96.0	1.9	2.0	98.2	1.9	96.3	2.3	2.2
Cotinine								
6.00 (LLOQ)	102.9	4.8	4.8	90.3	5.5	90.7	9.1	8.9
18.0	100.4	1.8	1.5	91.8	3.6	98.6	3.8	4.9
210	94.1	1.4	1.6	94.8	1.9	97.3	2.5	2.4
336	97.1	1.2	1.0	95.8	1.5	99.0	1.7	2.0
Hydroxycotinine								
6.00 (LLOQ)	103.4	5.7	3.9	109.9	4.5	103.5	12.6	8.3
18.0	102.0	5.7	3.5	101.9	6.6	109.1	2.8	5.9
210	99.9	3.3	2.5	103.1	1.2	105.6	5.1	4.1
336	100.0	3.3	3.8	106.1	1.6	105.0	2.9	3.6

Table 7
Limits of detection (LOD) and limits of quantitation (LOQ) for all analytes.

Analyte	LOD (ng/mL)	LOQ (ng/mL)
Nicotine	0.05	0.15
Cotinine	0.09	0.30
Hydroxycotinine	0.12	0.40

3.5. Stability under benchtop, freeze and thaw, and autosampler conditions and long-term storage stability in human plasma

Concentrations of analytes was within 85% – 115% of the nominal value for both QC levels under the tested conditions. Recoveries after short-term storage on ice ranged from 91.6% to 109.6% and at room temperature from 86.2% to 111.4% after 5 h. After the third freeze and thaw cycle, the recovery ranged from 94.6% to 108.2%. Recoveries of samples that were kept under autosampler conditions at 15 °C for 24 h

ranged from 99.2% to 113.8%. Further details and the complete data set are presented in the Supplementary Material (Tables S.2 – S.4).

As plotted in Fig. 6, all analytes remained stable over 119 days for both storage conditions, −80 °C and −20 °C. No increase of cotinine and hydroxycotinine was found over time, indicating that no further metabolism of nicotine took place during storing time. Thus, samples that have been already spiked with internal standards can be stored for at least 119 days at either −80 °C or −20 °C. Additionally, stability was assessed after 344 days of storage at −80 °C. Recoveries for nicotine, cotinine, and hydroxycotinine ranged from 94.7% to 102.9%, confirming stability for the extended time period. Concentrations of analytes were assessed directly at sampling day and again in one run at the last sampling day. The concentrations of supernatants that were sampled at day 0 did not decrease after storage at −80 °C for 119 days. This shows that supernatants after protein precipitation can be stored at −80 °C for at least 119 days prior to dilution with eluent A.

3.6. Example chromatograms and application to real sample

Nicotine and its metabolites cotinine and hydroxycotinine were quantified. Representative chromatograms derived from human blank matrix with and without addition of internal standards and from a matrix calibration sample are shown in Fig. 7. For reasons of clarity, analytes and internal standards are presented in separate parts. Human blank plasma was free of peaks for analytes and internal standards (Fig. 7 a and b). Blank plasma spiked with 5.00 ng/mL internal standards was found to be free of analyte peaks (Fig. 7 c and d). Spiking of analytes and internal standards to human blank plasma (5.00 ng/mL nicotine, 60.0 ng/mL cotinine, 60.0 ng/mL hydroxycotinine, 5.00 ng/mL internal standards) resulted in the chromatogram shown in Fig. 7 g and h. The chromatogram at LLOQ (0.50 ng/mL nicotine, 6.00 ng/mL cotinine, 6.00 ng/mL hydroxycotinine, 5.00 ng/mL internal standards) is shown in Fig. 7 e and f. Signal to noise ratio of nicotine was 16.6. For proof of concept, the method was applied to real plasma samples that were taken from a volunteer (male, 30 years old) during a smoking session of a combustible cigarette. A routine cigarette smoker drew 2 puffs per minute from a conventional cigarette for 5 min. Blood was collected before and at different time points during and after the smoking session. It was processed as described in Sections 2.2 and 2.4. Fig. 7i and j show chromatograms resulting from real plasma, sampled 8 min after the volunteer started the procedure. The analytes nicotine, cotinine, and hydroxycotinine were quantified as 7.94 ng/mL, 61.2 ng/mL, and 32.2 ng/mL, respectively. Quantitation of the metabolites cotinine and hydroxycotinine plays an important role in addition to the determination of nicotine. Their ratio, calculated by dividing the plasma concentration of hydroxycotinine by the plasma concentration of cotinine, can be used as a surrogate marker for CYP 2A6 metabolic activity which is the main enzyme for nicotine metabolism [7–10]. Previous studies have shown that rate of nicotine metabolism is a factor for success of some NRT, likely due to higher nicotine blood levels [7–10]. Slow metabolizers were found to have lower nicotine metabolic ratios compared to normal metabolizers. A cut-off level of < 0.31 for slow metabolizers and > 0.31 for normal metabolizers has been described in the literature based on their data set of 1246 participants [9]. The nicotine metabolic ratio derived from the test smoker in this real plasma sample was 0.53, above the exemplary cut-off value of 0.31. Accordingly, the test person was classified as a normal metabolizer. Information on nicotine metabolic ratio should be assessed in parallel to nicotine plasma concentrations since it provides additional information on the metabolic status without additional testing. Nicotine metabolism can potentially influence consumption pattern or nicotine kinetics of the studied product. Thus, an analytical method that is developed for determination of nicotine in plasma should ideally include the analytes cotinine and hydroxycotinine as well.

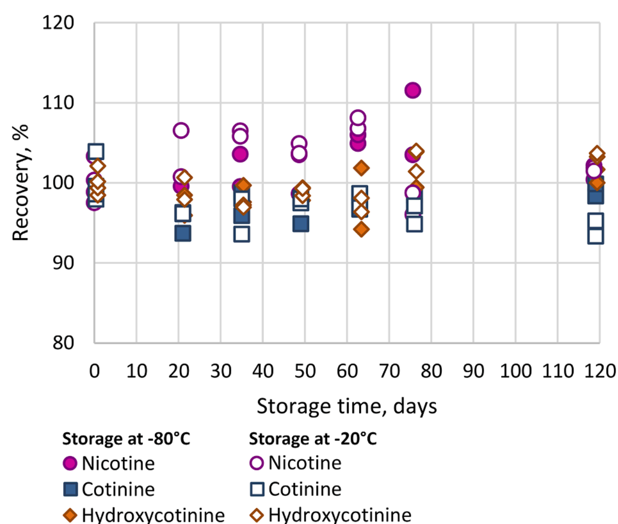

Fig. 6. Recovery of analytes after storage at −80 or −20 °C measured in one run at day 119.

3.7. Advantages of the method

The aim of this method development was to achieve high sensitivity for nicotine (LOQ < 0.5 ng/mL) without a time-consuming sample preparation procedure. Other well-documented methods for the determination of nicotine and/or its metabolites from biological matrices like blood plasma and urine include elaborate sample preparation protocols that are more complicated and time-consuming like solid-phase extraction [12,13,15,34–38] or liquid–liquid extraction [11,14,39–41] or even both [16]. However, sample handling time is only one aspect of many. The LOQ and the lowest level of the linear working range have to be suitable for the intended application. The main purpose of the method described herein is to quantify the rise in nicotine blood levels during use of nicotine delivery products in consumers. Since the volunteers will be asked to be abstinent from nicotine consumption overnight, blood level at t0 (directly prior to administration) are expected to be very low. Thus, the lowest level of the linear working range of nicotine should be 0.5 ng/mL. Some of the previously mentioned methods with a time-consuming sample clean-up step reported a LOQ for nicotine of 1 ng/mL or lower [11,12,15,16,35,37,41]. Yuan *et al.* performed protein precipitation and removed remaining matrix with online turbulent flow extraction prior to separation and reported a LOQ of below 0.5 ng/mL [42]. Another validated method combined protein precipitation with reverse phase chromatography, reporting a LOQ of 3 ng/mL [43]. The required high sensitivity for nicotine despite the high amount of remaining matrix constituents in the samples has been achieved due to prolonged retention of analytes in the herein described method. The first analyte nicotine elutes at 3.2 min while strong ion suppression due to matrix constituents has been present between 1.5 and 2 min. This extension of the retention time of nicotine to more than double than the solvent front time was accomplished by an increase of eluent pH value. At pH 4.50, the nitrogen atom in the pyridine ring of nicotine is predominantly uncharged leading to an enhanced interaction with the stationary phase. A further advantage of the herein described method is the low requirement for laboratory equipment. No special apparatus is needed for sample clean-up and the method runs stably on an older generation mass spectrometer (4000er series). If required for the study, sensitivity can possibly be increased further by switching to a newer generation mass spectrometer.

4. Conclusion

Protein precipitation is a very simple and rapid sample preparation technique with a minimum amount of sample handling time as well as

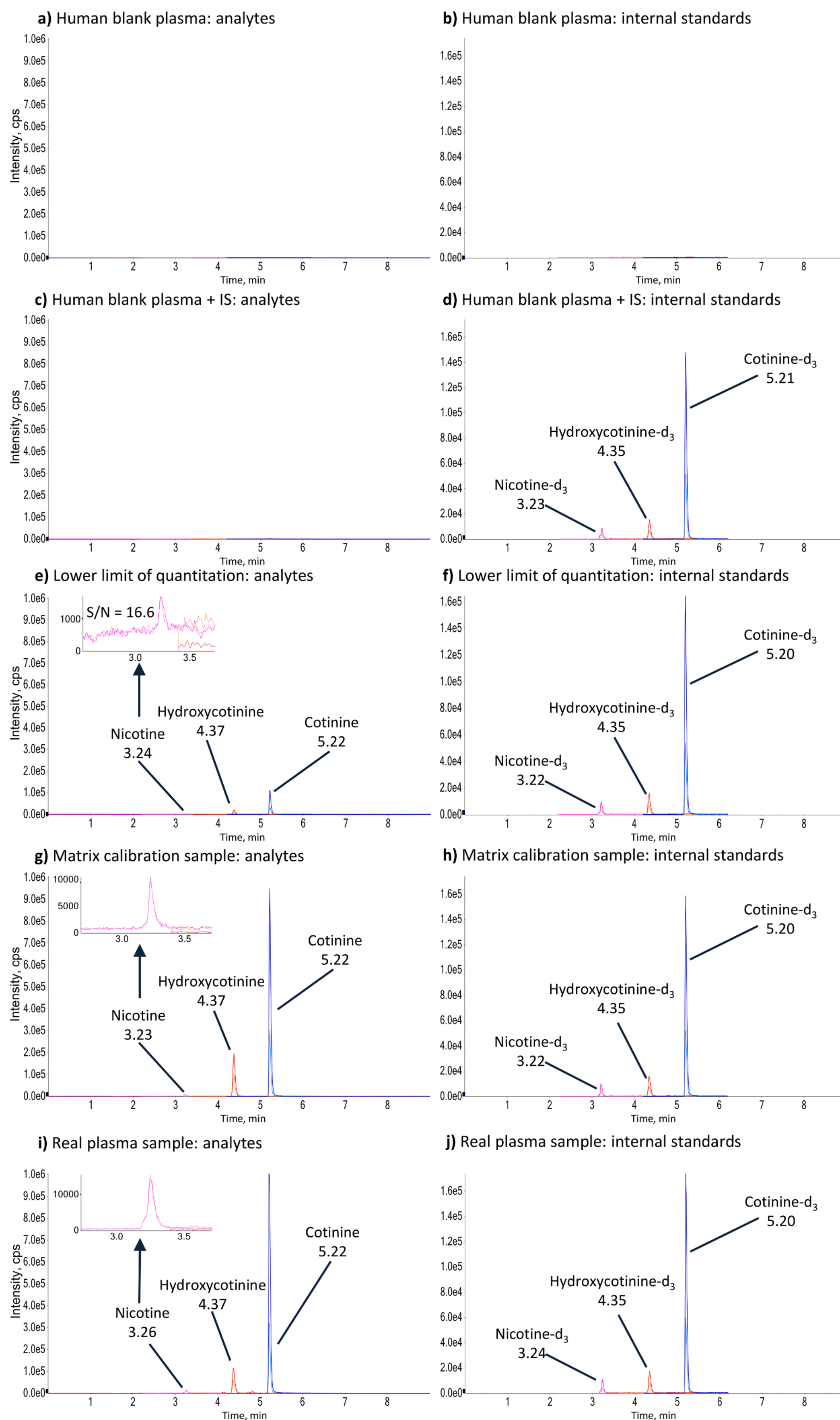

Fig. 7. Example chromatograms with quantifier and qualifier traces of analytes (a, c, e, g, i) and internal standards (b, d, f, h, j) in **a)** and **b)** human blank plasma, **c)** and **d)** human blank plasma spiked with 5.00 ng/mL internal standards, **e)** and **f)** LLOQ (0.50 ng/mL nicotine, 6.0 ng/mL cotinine and hydroxycotinine, 5.00 ng/mL internal standards), integrated zoom for the nicotine signal (signal to noise ratio 16.6), **g)** and **h)** matrix calibration sample (5.00 ng/mL nicotine, 60.0 ng/mL cotinine and hydroxycotinine, 5.00 ng/mL internal standards), integrated zoom for the nicotine signal, **i)** and **j)** real plasma sample from a smoking volunteer, integrated zoom for the nicotine signal. For all analytes but cotinine and all internal standards, the quantifier trace shows the higher signal.

sample amount needed. Human plasma was mixed with cold methanol to precipitate proteins. After centrifugation, the supernatant was diluted with aqueous eluent A to reduce the amount of methanol prior to injection into the LC-ESI-MS/MS system. This fast, robust, and sensitive procedure allows a high throughput of samples. Remaining matrix after protein precipitation can potentially interfere with the ionization of co-eluting analytes and thus reduce sensitivity. Improved retention of analytes can separate elution and ionization of analytes from early

eluting matrix components and consequently reduce ion suppression. Thus, a phenyl-hexyl stationary phase was selected, and mobile phase composition was optimized to improve π-π interactions between stationary phase and analytes. A pH value of 4.50 was selected for aqueous eluent A to avoid protonation of the pyridine ring of nicotine. As organic eluent B, methanol was shown to be superior to acetonitrile. Ion suppression of co-eluting matrix components was assessed with a post-column infusion setup and confirmed to be low. The resulting LC-ESI-MS/MS method for quantitation of nicotine and its most important metabolites cotinine and hydroxycotinine in human plasma was validated with a linear working range of 0.50–35.0 ng/mL for nicotine and 6.00 to 420 ng/mL for cotinine and hydroxycotinine. The method was shown to be selective, sensitive, reproducible, repeatable, and rapid with an easy sample preparation step. Application to real plasma samples of a smoking volunteer was successful. The herein described protocol will be used in an ongoing study on nicotine delivery by electronic cigarettes and may be adopted by other laboratories with similar projects.

CRediT authorship contribution statement

Nadja Mallock: Conceptualization, Investigation, Validation, Writing - original draft, Methodology. **Andrea Rabenstein:** Conceptualization. **Peter Laux:** Conceptualization, Writing - review & editing. **Tobias Rüther:** Conceptualization. **Christoph Hutzler:** Supervision, Conceptualization, Writing - review & editing, Validation. **Maria Kristina Parr:** Supervision, Conceptualization, Writing - review & editing. **Andreas Luch:** Supervision, Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2021.122736.

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