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RESEARCH PAPER

High-throughput wide dynamic range procedure for the simultaneous quantification of nicotine and cotinine in multiple biological matrices using hydrophilic interaction liquid chromatography-tandem mass spectrometry

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Abstract A straightforward, high-throughput method was developed and fully validated for the simultaneous determination of the specific tobacco biomarkers nicotine and its main metabolite cotinine in a wide dynamic range and supporting the most common human biological matrices (urine, oral fluid and hair). Sample preparation was performed inside the very HPLC injection vials by pipetting 0.5 mL of the liquid samples, deuterated internal standards in alkaline solution and dichloromethane as extraction solvent. Solid samples (i.e. around 10 mg hair) were first submitted to alkaline digestion in the HPLC vials and processed accordingly. The organic phase (reached through the upper aqueous layer) was directly injected without further treatment. Instrumental analysis was performed using hydrophilic interaction (HILIC) ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Total chromatographic time was 2 min. The method covers a wide dynamic range making it fit-for-purpose for the analysis of samples covering entire populations, irrespective of the level of exposure or tobacco use. Calibration curves (r^2>0.995) covered the range 1–2000 ng/mL (or 0.05–100 ng/mg hair) for nicotine and 0.1–2000 ng/mL (or 0.005–100 ng/mg hair) for cotinine. Within-run and between-run precision and accuracy were typically below 10 %, and always below 20 % at the lower limit of quantification. The method was successfully applied to the analysis of samples from different projects involving multiple matrices.

Keywords Nicotine · Urine · Saliva · Hair · Tobacco biomarkers · LC-MS/MS

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Introduction

Tobacco consumption is the leading cause of cancer death worldwide. It may also be the origin of cardiovascular and respiratory diseases, including acute myocardial infarction and chronic obstructive pulmonary disease [1]. Passive exposure to second hand smoke (SHS) also increases the risk of lung cancer and acute myocardial infarction in non-smokers [2]. The ratification of the WHO Framework Convention on Tobacco Control [3] requires all signatory countries to adopt and implement effective legislative measures to protect the population from exposure to tobacco smoke in indoor workplaces, public transport, indoor public places and, as appropriate, other public places. As a consequence, smoke-free laws have regulated the places where tobacco products can be used

[4]. There is an increasing interest in monitoring the use of tobacco products and the exposure to SHS in larger number of samples, encompassing whole populations. Lower concentrations were also expected as non-smokers became the target of many studies [5]. Using biomarkers to get a quantitative measurement of the real exposure to tobacco smoke is very relevant and much more accurate than self-reported data of the perceived exposure obtained through questionnaires [6–8].

Nicotine and its main metabolite cotinine have been extensively used as specific markers of tobacco exposure. Depending on the aim of the study, different matrices have been chosen, being the non-invasive, e.g. urine, oral fluid and hair the most widely analysed [9].

Cotinine, with a half-life of 7–40 h, is the preferred biomarker in urine and oral fluid. Nicotine is found in much lower concentrations and has a much shorter half-life of 2–3 h. Urine and oral fluid concentrations give a good indication of the exposure in the 3 or 4 days prior to sample collection [10].

Hair grows approx. 1 cm per month, on average. Segmental analysis allows measuring the level of exposure along months, depending on the length of the hair shaft obtained [11, 12]. Nicotine in hair has been traditionally preferred over cotinine owing to its much higher concentrations [13].

Fast and straightforward quantification of nicotine and cotinine in multiple biological matrices is needed. Furthermore, procedures should be applicable to a wide range of concentrations covering samples from heavy smokers to non-smokers or even newborns with little exposure [14].

There is abundant literature on the measurement of nicotine and cotinine in different biological matrices ranging from radioimmunoassays [10, 15] to gas and/or liquid chromatography methods, with a wide variety of detectors [16–23]. In the last years, tandem mass spectrometry has become the detection system of choice [14, 17, 18, 24–28].

Sample preparation procedures using either solid-phase extraction or liquid-liquid extraction have been described with varying complexity depending on the selectivity and type of instrumental system used. Limits of quantification (LOQ) as low as 0.02 ng/mL of cotinine in oral fluid or urine [5, 29] and 0.04 ng/mg of nicotine in hair [30] have been described. However, all procedures required steps that were throughput bottlenecks. A new approach was required to reach a balance between the easiest sample preparation for most biological matrices and compatibility with chromatographic conditions and instrumental sensitivity.

The aim of the present work was the development of a high-throughput quantitative procedure for the most commonly used tobacco biomarkers (i.e. nicotine and cotinine), applicable to urine, oral fluid and hair analysis.

Experimental

Chemicals

(−)-Nicotine and (−)-cotinine 1.0 mg/mL standard solutions in methanol as well as HPLC grade of formic acid were purchased from Fluka-Sigma-Aldrich (Madrid, Spain). (±)-nicotine-d_4 (2,4,5,6-tetradeutero-3-(1-methylpyrrolidin-2-yl)-pyridine) 100 μg/mL solution in acetonitrile and (±)-cotinine-d_3 (5-(3-pyridinyl)-1-trideuterometyl-2-pyrrolidinone) 1.0 mg/mL in methanol, were purchased from Cerilliant Corp (Round Rock, Texas, USA). HPLC grade methanol and acetonitrile, as well as analytical grade sodium hydroxide, potassium chloride and ammonia solution 25 % were obtained from Merck Millipore (Darmstadt, Germany). HPLC-grade dichloromethane was purchased from Scharlau (Barcelona, Spain). Ultrapure water was obtained from a Millipore Milli-Q water purification system.

Biological sample collection

Human blank specimens (urine, oral fluid and hair) for the development and validation of the method were obtained from healthy donors at IMIM (Hospital del Mar Medical Research Institute), Barcelona (Spain). Oral fluid specimens were collected as part of a cross-sectional study [25, 31, 32] in partnership with the Catalan institute of Oncology (ICO). It was a representative sample of the general population of Barcelona (Spain) (n=1245) consisting of smokers and non-smokers exposed to SHS. Urine plus oral fluid and hair samples (n=49 each) were also collected from a convenience sample [33] (funded by Instituto de Salud Carlos III-FEDER, Government of Spain, grants PI081339 and PI081436). Finally, hair samples were also collected from different cohorts of newborn and infants (n=629) with smoker parents (a project from the Spanish National Committee on Smoking Prevention, grant CNPT0701) [34]. Hair was collected from the vertex posterior where possible. Protocols were approved by the respective Local Ethics Committees and all participants provided written informed consent. Oral fluid samples were collected by spontaneous generation helped by sucking a lemon candy (Smint®). Liquid matrices were kept at −20 °C. Hair samples were cut close to the scalp, and kept in individual envelopes at room temperature with both ends (proximal and distal) identified.

Preparation of standard solutions

Nicotine and cotinine 1.0 mg/mL solutions were used, as purchased, as primary stock solutions. Separate working solutions (*calibration curve* and *quality controls* from different product batches) were prepared by proper dilutions in acetonitrile at concentrations of 0.1, 1, 10 and 100 μg/mL for

nicotine and 0.01, 0.1, 1, 10, 100 μg/mL for cotinine. All stock and working solutions were stored at −20° in amber glass screw-capped bottles.

Preparation of internal standard solutions

For nicotine-d_4, the purchased 100 μg/mL solution was used as stock solution. For cotinine-d_3, a 100 μg/mL solution was prepared as stock solution by diluting 10 times the original 1 mg/mL solution with acetonitrile.

Two different internal standard (IS) working solutions containing both substances were prepared. For the analysis of liquid matrices (urine, oral fluid or water) 250 μL of the nicotine-d_4 plus 25 μL of the cotinine-d_3 stock solutions were diluted to 250 mL with a NaOH 1 M, KCl 2 M aqueous solution. For hair analysis, a further six-fold dilution of the previous solution was used. These working solutions were kept at 4 °C in an amber bottle and could be used for up to 3 months.

Sample preparation

In an HPLC injection vial (high recovery conical bottom 1.5 mL, Agilent ref. no. 5184–3551), 0.5 mL aliquot of a liquid sample (urine, centrifuged oral fluid or water), 0.1 mL of the corresponding IS working solution (see above) and 0.5 mL dichloromethane were added. Vials were crimped, placed in a rocking mixer for 15 min at a frequency of 50 min^{-1} and centrifuged at 3400×g for 10 min. Hair samples were first washed (three times with dichloromethane by sonication for 10 min). After drying at a temperature below 40 °C, the segment to be analysed was put in a flat bottomed plastic tubes (50×16 mm), finely cut with scissors and kept at room temperature until analysis. In an HPLC injection vial, an amount of approx. 10 mg was directly weighed and 0.6 mL of the corresponding IS working solution added. Vials were crimped and kept for 30 min at 80 °C in a dry block. The resulting digested samples were extracted as the liquid matrices above. Vials were stored at −20 °C until analysis.

Liquid chromatography-tandem mass spectrometry

Analyses were performed on an Agilent Technologies LC 1200 series HPLC system connected to an Agilent 6410 triple quadrupole mass spectrometer, through an electrospray ionization source working in positive ionization mode. Chromatographic separation was achieved using an ultra-rapid hydrophilic interaction chromatography column Waters Acquity UPLC® BEH HILIC 50 mm long, 2,1 mm I.D. 130 Å pore, 1.7 μm particle size. The column temperature was kept at 35 °C. The needle of the injector was externally rinsed with methanol/water (1:1) for 10 s prior to each injection. A volume of 10 μL of the organic layer of each extracted sample (reached through the aqueous upper layer) was directly injected for analysis. The auto-sampler tray was kept at 4 °C.

A binary gradient of (A) 10 mM aqueous ammonium formate solution adjusted to pH 3 with formic acid and (B) acetonitrile, at a flow rate of 0.6 mL/min was used. The gradient increased from 5 % A to 40 % A in 1.5 min and decreased to 5 % A over another 0.5 min (total run time 2 min). MS source conditions were as follows: capillary voltage (positive), 4000 V; desolvation gas temperature, 300 °C; drying gas flow, 6 L/min; nebulizer pressure, 15 psi. High-purity nitrogen (99, 999 %, Abello-Linde, Spain) was used as collision gas. As nebulizer and drying gas, nitrogen was obtained from a central high flow permanent supply using a liquid nitrogen bulk tank (99.5 %, Praxair, Spain).

MS/MS parameters were optimized by injecting 10 μL of 10 ng/mL individual standard solutions in dichloromethane using the final chromatographic conditions including column, gradient, etc. An automated process using Optimizer for 6400 Series Triple Quadrupole version B.06.00 from Agilent was used for method development. Confirmation of the final conditions was done by repeated injections bracketing the suggested optimal set-up. Fragmentor voltage at 135 V, collision energy 20 V and dwell time 100 ms were chosen in all cases. The MRM transitions for quantification and identification were respectively m/z 163 to 130 and 117 for nicotine, m/z 167 to 134 and 121 for nicotine-d_4; m/z 177 to 80 and 98 for cotinine and m/z 180 to 80 and 101 for cotinine-d_3. Data were acquired and processed using MassHunter Quantitative Analysis v B.06.00.

Calibration and quality control samples

After validation of the equivalence between calibration curves prepared in water versus those prepared in each biological matrix, routine calibration curves were prepared in 0.5 mL water aliquots as follows:

Six point calibration curves were prepared in duplicate containing 1, 100, 500, 1000, 1500 and 2000 ng/mL for nicotine and 0.1, 100, 500, 1000, 1500 and 2000 ng/mL for cotinine. Additionally, blank samples and blank samples devoid of IS were also included.

Quality control samples (low, medium and high QCs) were prepared containing 1.2, 800, 1700 ng/mL nicotine and 0.12, 800, 1700 ng/mL cotinine respectively. QCs were prepared in different samples of each matrix all through the validation protocol. Hair samples (10 mg) were spiked with the same amount per vial as the 0.5 mL liquid samples.

Method validation in water, urine, oral fluid and hair

Validation followed a four-assay protocol in line with the current EMEA Guideline on bioanalytical method validation [35]. The following parameters were studied: specificity,

selectivity, limit of detection and quantification, linearity, dilution integrity, precision, accuracy, recovery, carryover, matrix effect and short- and long-term stability. The effect of the sample amount was also tested for the solid matrix (hair). Calibration curves were prepared in 10 mg hair and 0.5 mL aliquots of water, urine and centrifuged oral fluid to cross-validate their equivalence.

Specificity was assessed by analysing six blank specimens of each matrix from different individuals with and without IS. Selectivity was tested with respect to other nicotine metabolites (i.e. nornicotine, norcotinine, 3′-hydroxycotinine, nicotine *N*-oxide, cotinine *N*-oxide and 4-hydroxy,4-(3-pyridyl)-butanoic acid).

The limit of detection (LOD) and limit of quantification (LOQ) were calculated as the concentrations giving a signal to noise ratio of 3.3:1 and 10:1 respectively. The noise was estimated as the standard deviation of the signal (area of analyte divided by area of the IS) obtained from all replicates (n= 4) of the lowest calibrator (lowest limit of quantification, LLOQ). It was verified that the LLOQ had a value above LOQ for all matrices, showing its suitability.

Linearity of the method was tested along four assays by calculating the weighted least square regression line, using $1/x$ as the weighting factor. The first assay was performed running four replicates at each concentration and curves prepared in each biological matrix. A Dixon test was used to test for outliers. The rest of the curves were performed in duplicate. The determination coefficient r^2 was required to be greater than 0.995 for each analyte. Calibrators were required to be within ±15 % when calculated against the curve except for the LLOQ for which ±20 % was allowed. The 95 % confidence interval for the difference of both slopes and intercepts were computed and compared for equivalence between different matrices. Following a cross-validation approach, calibration samples and QCs prepared in each matrix were back-calculated using the calibration curve in water to test for the adequacy of the method.

Dilution integrity was tested by spiking blank matrices at double the high QC (i.e., 3400 ng/mL) and diluting the sample as needed. For the solid matrix, the effect of sample amount was tested by homogenising a hair sample from a smoker and then analysing aliquots 2, 5, 10 or 50 mg.

Within-run precision (repeatability) was expressed as the coefficient of variation (%CV) of the calculated concentrations of the five replicates of each quality control sample (n=5) analysed on the same batch. Between-run (intermediate) precision was calculated from all replicates of each quality control along the 4-day protocol (n=20).

Accuracy was calculated as the error, expressed as percent of the nominal concentration (%error), obtained for the quality control samples. A maximum deviation of±15 % from the nominal value (20 % at the LLOQ) was accepted.

Extraction recovery was calculated as the ratio between the mean peak area of the analytes obtained from samples spiked before and after extraction (separating and spiking the organic phase) [36] using four different matrices at three concentrations. Matrix effect was expressed as the ratio between the mean peak area obtained from extracts corresponding to 100 % recovery (see above) and standard solutions prepared in dichloromethane at the same concentrations. The overall recovery (or process efficiency) was calculated by multiplying extraction recovery by matrix effect.

Carryover was tested by analysing blank samples immediately following spiked samples. To rule out any impact on method performance due to the direct injection of the organic phase without separation of the upper aqueous phase, 10 consecutive injections of the same sample in each matrix were performed. System pressure and full scan profiles were monitored.

Method-related stability was tested as follows:

Short-term (within-run) stability was evaluated along the validation protocol by properly distributing QC samples along the sequence of the analytical batches and applying an analysis of variance using the area of the analyte as the independent variable, the amount as factor and the time of analysis (since the beginning of the analytical batch) as covariate. A significance p>0.05 was used to reject a correlation, therefore proving that samples were stable under within-run conditions.

Long-term stability was tested by analysing QC samples immediately after preparation or after 4 weeks stored at −20 °C.

The effect of freeze-thaw cycles (n=3) on the extracted QC samples was tested by analysing the extraction vials after submitting them to up to three consecutive freeze-thaw operations (one per day) and analysed all together on a single run at the end of the protocol.

Results

Figure 1 shows the full product ion spectra of nicotine and cotinine as well as the proposed structure of their fragment ions. Structures were elucidated using spectra from isotopically labeled analogues and they clarify some discrepancies found in the literature [37–40].

Specificity, or selectivity for endogenous interferences, was tested with six specimens of each matrix. No sample exceeded the LOD for the analytes of interest.

Selectivity of the assay with respect to other nicotine metabolites was thoroughly tested by concurrently analysing them and testing for their retention times, mass spectra and extraction recovery. Norcotinine, sharing the same molecular mass of nicotine, was a potential interference. However, it

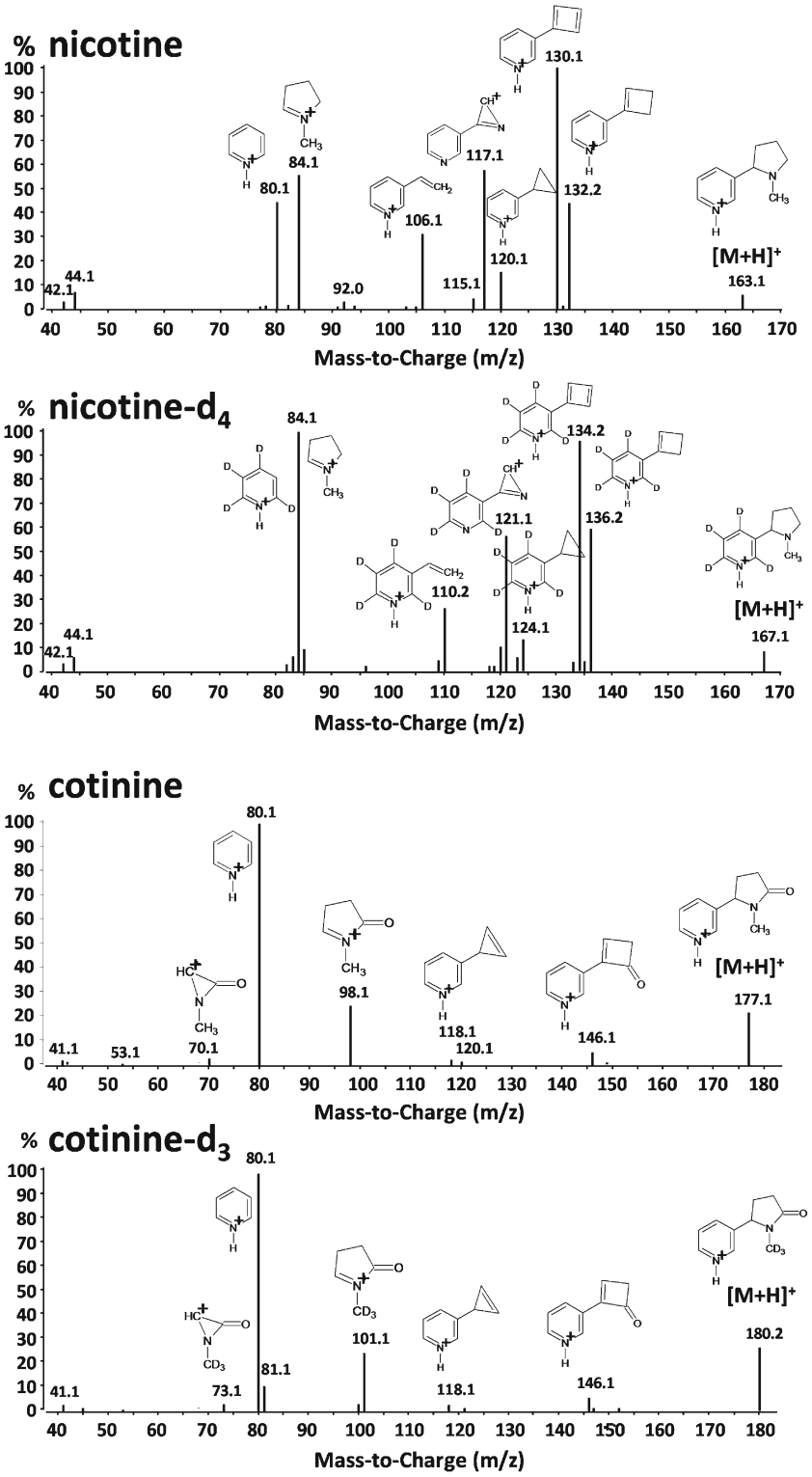

Fig. 1 Product ion mass spectra obtained for nicotine (precursor ion $[M+H]^+$ at *m/z* 163), cotinine (precursor ion $[M+H]^+$ at *m/z* 177) and their corresponding deuterated analogues used as internal standards, nicotine-d_4 (precursor ion $[M+H]^+$ at *m/z* 167) and cotinine-d_3 (precursor ion $[M+H]^+$ at *m/z* 180)

eluted right after cotinine (RT = 0.61 min), completely resolved from nicotine. Other metabolites did not share the molecular mass of any of the analytes or their retention times. The poor extraction efficiency of the most polar metabolites (e.g. 3′-hydroxycotinine or 4-hydroxy-4-(3-pyridyl)-butanoic acid) needs also to be considered in terms of selectivity.

Linearity was proven through the whole calibration range and for all matrices with determination coefficients (r^2) always

Table 1 Calibration curve parameters (mean±standard error), limit of detection (LOD) and quantification (LOQ) obtained for the analysis of nicotine and cotinine in biological matrices (oral fluid, urine and hair) and in water. For comparison, amount of analyte (ng) rather than concentration was used for the regression analysis

Analyte	Matrix	LOD (ng)	LOQ (ng)	LOQ (ng/mL[a])	Slope	*y*-intercept	r^2
Nicotine	Water	0.046	0.14	0.28	0.1332±0.0008	−0.0049±0.0281	0.9993±0.0794
	Oral fluid	0.079	0.24	0.48	0.1294±0.0011	−0.0043±0.0354	0.9988±0.0099
	Urine	0.050	0.15	0.30	0.1295±0.0016	−0.0085±0.0560	0.9973±0.1582
	Hair	0.086	0.26	0.026	0.1255±0.0011	−0.0105±0.0396	0.9985±0.1120
Cotinine	Water	0.0089	0.027	0.054	0.9214±0.0117	0.0155±0.0846	0.9975±0.1070
	Oral fluid	0.0066	0.020	0.040	0.9963±0.0150	0.0091±0.1090	0.9962±0.1379
	Urine	0.0086	0.026	0.052	1.0091±0.0811	0.0029±0.0810	0.9988±0.1731
	Hair	0.0066	0.020	0.0020	0.9311±0.0212	0.0087±0.1676	0.9969±0.2120

[a] For hair samples, LOQ corresponds to ng/mg (for a 10 mg sample)

higher than 0.995. Table 1 shows the results obtained including the calculated values of LOD and LOQ. For the sake of comparison, regression parameters are given using amount (ng spiked to the aliquot of sample) instead of concentration, as it would change between liquid and solid matrices. The confidence intervals (IC95%) of the difference between slopes or intercepts of the calibration curves in each matrix showed not being statistically significant ($p>0.05$). Concentration of calibration samples, as well as quality control samples in each matrix prepared through the four validation protocol, were back-calculated using the calibration curve in water. Accuracy (%error) was always below 15 % (±20 % for calibration curves at LLOQ) showing the adequacy of the calibration curve prepared in water.

Extraction recovery was above 90 % for both analytes in all matrices. The overall process efficiency (which will indicate mainly the matrix effect) is summarized in Table 2. Urine gave lower results (i.e. around 60 %). Oral fluid and hair were in the range 65–90 %. These differences were well corrected by the IS and had no impact on the LLOQ chosen. Dilution integrity was proven and the analysis of increasing amounts (2, 5, 10 or 50 mg) of the same homogeneous non-blank hair sample gave a result of 2.6± 0.3 ng/mg nicotine and 0.15±0.01 ng/mg cotinine with no statistical correlation between individual values and the amount of sample taken for analysis.

Within-run and between-run precision, expressed as the CV% of each QC sample concurrently analysed on the same batch are given in Table 3. Accuracy, expressed as %error, is given in Table 4. Values were consistent with the requirements.

Carryover was found to be 0.4 % for nicotine and 0.3 % for cotinine. These values were also obtained when injecting pure standard solutions in dichloromethane, therefore not linked with the extraction procedure. Samples were not contaminated as repeated analysis after testing for carryover resulted in clean blank samples. The theoretical contribution of the carryover was automatically calculated for each sample analysed in a run. When this contribution was higher than 10 % of the calculated concentration, the sample was re-injected. LODs and LOQs are given in Table 1. These values proved that the lowest calibrator was well suited as LLOQ for quantification of samples of any matrix.

The test for within-run stability proved that there was no significant difference in the results obtained for the QC samples with respect to the time until analysis and at any of the concentrations tested. Long-term stability was also proven with quantitative differences after 1 month at −20 °C being within the accuracy tolerance. No statistical difference was found between the results obtained when analysing samples after up to three freeze-thaw cycles.

Table 2 Overall recovery (process efficiency) for the analysis of nicotine and cotinine in biological matrices (oral fluid, urine and hair) as well as in water (mean±SD)

Analyte	Matrix	Process efficiency (%, *N*=4)		
Nicotine		1 (ng/mL[a])	500 (ng/mL[a])	2000 (ng/mL[a])
	Water	82.8±2.8	87.3±5.0	87.4±2.2
	Oral fluid	77.9±2.1	75.9±3.2	76.1±4.0
	Urine	68.2±1.1	67.3±1.3	66.1±0.9
	Hair	70.6±2.5	79.0±5.1	90.2±3.6
Cotinine		0.1 (ng/mL[b])	500 (ng/mL[b])	2000 (ng/mL[b])
	Water	89.5±3.9	106.7±1.9	91.0±1.8
	Oral fluid	65.2±4.0	81.3±6.6	67.8±1.3
	Urine	59.4±3.7	60.4±2.8	58.4±1.6
	Hair	68.7±5.5	82.9±4.4	77.2±0.4

[a] For 10 mg hair samples, nicotine concentrations correspond to 0.05, 25 and 100 ng/mg respectively

[b] For 10 mg hair samples, cotinine concentrations correspond to 0.005, 25 and 100 ng/mg respectively

Table 3 Within-run and between-run precision for the analysis of nicotine and cotinine in biological matrices (oral fluid, urine and hair) as well as in water expressed as their coefficient of variation (CV%). Quantification was performed using calibration curves extracted from water

Analyte	Matrix	Within-run (%CV, N=5)			Between-run (%CV, N=20)		
		Low	Medium	High	Low	Medium	High
Nicotine	Water	4.3	2.2	1.7	8.4	2.8	2.6
	Oral fluid	2.8	1.9	2.7	7.9	1.5	4.9
	Urine	2.8	2.7	3.1	12.1	2.7	3.4
	Hair	5.3	1.7	3.7	5.9	4.3	3.5
Cotinine	Water	18.7	3.4	5.0	16.1	5.8	5.5
	Oral fluid	13.0	2.6	6.0	10.3	3.0	6.1
	Urine	5.9	2.6	6.5	17.2	4.9	6.1
	Hair	10.5	4.2	5.7	11.3	5.7	5.4

The application of the method to the analysis of cotinine in oral fluid collected from a representative sample of the general population of Barcelona (n=1245) showed a mean concentration of 58±130 ng/mL. Interestingly, the subpopulation that declared being non-exposed non-smokers (n=673) showed a mean concentration of 1.7±17 ng/mL, with the highest value being 19 ng/mL. Those declaring being exposed non-smokers (n=223) had a mean cotinine concentration of 2.9±15 ng/mL with the highest value being 160 ng/mL (a value well inside the smoker's range). Nicotine concentrations, not regularly monitored in saliva because of the risk of contamination in smokers, were higher in all cases. Urine samples were also analysed in a sub-group of non-smokers (n=49) with a cotinine mean concentration of 2.2±5.1 ng/mL.

Hair samples were also analysed from 629 newborn and children younger than 6 months of age with smoking parents. The mean nicotine concentration found was 6.0±9.1 ng/mg with the highest being 83 ng/mg (a concentration well inside the range expected for a smoker). Mean cotinine concentration was 0.24±0.38 ng/mg. Figure 2 shows representative chromatograms of the analysis of samples from individuals with different levels of exposure to tobacco smoke as well as calibration and control samples.

Discussion

The need for high-throughput methodologies has been fostered by the bioanalytical requirements of epidemiological studies [41]. Nicotine and its main metabolite cotinine are the preferred biomarkers of environmental tobacco smoke exposure [6], so they were chosen for this work. Increasing sensitivity and applicability to different matrices are also requirements imposed by the decreasing levels of exposure.

Cotinine, and particularly nicotine, are polar compounds that performed well under hydrophilic interaction chromatographic conditions. Sensitivity showed to improve by an order of magnitude with respect to equivalent reverse phase conditions, under high acetonitrile content and the presence of an ammonium buffer. The use of ultra-high performance liquid chromatography allowed shortening analysis time down to 2 min. Cotinine and nicotine show proper retention and good peak shape: 0.45 min (k=2.1) and 1.46 min (k=6.0) respectively. Extraction was performed in the very HPLC vials. The elevated organic content of the mobile phase allowed direct injection of the extraction solvent obviating further evaporation steps. Dichloromethane offered multiple advantages. Recoveries were excellent under the alkaline and salting-out

Table 4 Within-run and between-run accuracy for the analysis of nicotine and cotinine in biological matrices (oral fluid, urine and hair) as well as in water expressed as the mean % difference of the value obtained with respect to the nominal value (%error). Quantification was performed using calibration curves extracted from water

Analyte	Matrix	Within-run (%error, N=5)			Between-run (%error, N=20)		
		Low	Medium	High	Low	Medium	High
Nicotine	Water	3.4	2.0	−1.4	−3.3	2.7	−2.1
	Oral fluid	16.7	0.4	−4.7	10.6	0.6	−2.1
	Urine	7.1	1.3	1.5	11.1	0.0	0.7
	Hair	−1.3	4.2	−1.0	4.2	1.5	−0.4
Cotinine	Water	−5.5	1.0	−0.3	−8.1	5.0	−2.3
	Oral fluid	−6.6	7.3	6.2	−8.2	6.5	3.0
	Urine	0.5	10.1	7.5	9.7	6.7	5.0
	Hair	−5.6	9.8	−5.8	−1.0	5.5	−3.1

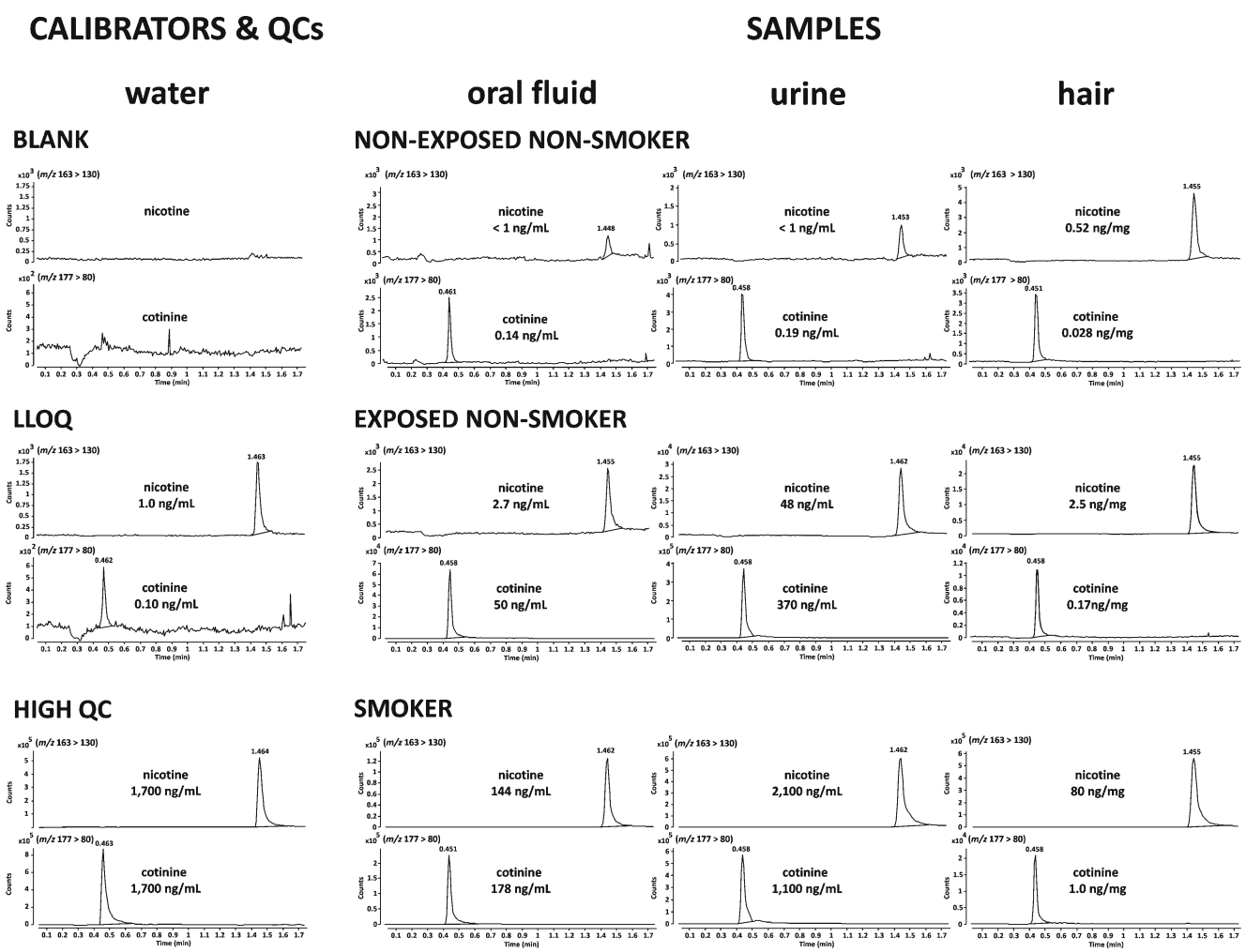

Fig. 2 Representative chromatograms corresponding to the analysis of samples of a non-exposed non-smoker, an exposed non-smoker and a regular smoker analysed as well as calibration and quality control samples extracted from water

effect conditions used. The HPLC injector needle reached the lower dichloromethane phase through the upper aqueous layer without any noticeable impact on background or carryover. It also helped in preventing evaporation and extending the stability of extracted samples.

Regarding mass spectra of nicotine and cotinine, publications have shown logical differences depending on the instrument used. However, the structural elucidation of the fragment ions produced lacks some consistency [37–40, 42–44]. The spectrum of nicotine-d_4 shows fragments at *m/z* 136 and 134 (analogues to the fragments at *m/z* 132 and 130 of nicotine-d_0) proving that those structures cannot be attributed to the frequently described quinoline like cyclizations [38–40]. Complementary, spectra obtained for [3′,3′-d_2]-nicotine [37] explains the presence of a double bond in position 2′ justifying the structure of its main fragment at *m/z* 130. In choosing equivalent fragments between analytes and IS analogues, the pyridine fragment at *m/z* 80 shall be avoided when using deuterated analogues at that ring, as their corresponding fragment at *m/z* 84 shares the mass with the pyrrolidine ring resulting in an apparent change in behaviour between analyte and IS (see Fig. 1).

As in previous publications, the method showed good linearity over a wide dynamic range [28, 44, 45], making it suitable for the analysis of samples from very different tobacco exposure patterns. LOQs were well fit for purpose. Samples showed quantifiable amounts, except for some belonging to self-declared non-exposed individuals. Amounts in the range 1–10 % of those found in exposed individuals have been described as potentially coming from the diet [10]. Those amounts are consistent with the LLOQs chosen and samples from real non-exposed individuals resulted in non-quantifiable concentrations. Tests performed using a lower dichloromethane volume (250 μL) showed to work well, increasing the analyte concentration and improving the LOQ. Selectivity with respect to endogenous interferences was proven. However, some blank samples had to be discarded as they showed to contain detectable amounts of nicotine and cotinine. Guidelines for method validation encourage the use of calibrators and QCs in the matrix to be analysed [35,

46–49], although they also provide for the possibility of using surrogate matrices when they are difficult to obtain. A key element of this multi-matrix development was the cross-validation of the suitability of calibration curves extracted from water for the quantification of samples in any of the three matrices assayed. It simplified and favored the applicability of the method for the analysis of any sample. Apart from oral fluid, urine and hair, for which a thorough validation was performed, preliminary tests performed in serum and plasma also showed equivalent good results.

There is abundant literature on the detection and/or quantification of many other nicotine metabolites for different reasons [18, 28, 45]. Many of those metabolites are equally well extracted with dichloromethane and can be readily incorporated to the current method. Others, particularly those more polar (i.e. 3′-hydroxycotinine, etc.) are poorly extracted from pure dichloromethane. Mixtures containing isopropanol have been frequently used either for liquid-liquid or solid-phase extraction. Those more polar solvents would not be compatible with the HILIC conditions used.

Conclusion

The quantitative procedure developed minimizes sample preparation steps by extracting the samples in the very injection vials. Separation using ultrafast liquid chromatography reduces chromatographic time down to 2 min. It is suitable for the analysis of nicotine and cotinine in multiple biological matrices (oral fluid, urine and hair) using a common calibration curve extracted from water. Due to its proper LLOQs and wide dynamic range, the procedure is well suited for the analysis of samples from entire populations, from heavy smokers to subjects with low-level exposure to environmental tobacco smoke.

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Conflict of interest The authors declare no conflict of interest.

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