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Determination of nicotine and nicotine metabolites in urine by hydrophilic interaction chromatography–tandem mass spectrometry: Potential use of smokeless tobacco products by ice hockey players

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ABSTRACT

Consumption of nicotine in the form of smokeless tobacco (snus, snuff, chewing tobacco) or nicotine-containing medication (gum, patch) may benefit sport practice. Indeed, use of snus seems to be a growing trend and investigating nicotine consumption amongst professional athletes is of major interest to sport authorities. Thus, a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the detection and quantification of nicotine and its principal metabolites cotinine, *trans*-3-hydroxycotinine, nicotine-*N′*-oxide and cotinine-*N*-oxide in urine was developed. Sample preparation was performed by liquid–liquid extraction followed by hydrophilic interaction chromatography–tandem mass spectrometry (HILIC–MS/MS) operated in electrospray positive ionization (ESI) mode with selective reaction monitoring (SRM) data acquisition. The method was validated and calibration curves were linear over the selected concentration ranges of 10–10,000 ng/mL for nicotine, cotinine, *trans*-3-hydroxycotinine and 10–5000 ng/mL for nicotine-*N′*-oxide and cotinine-*N*-oxide, with calculated coefficients of determination (R^2) greater than 0.95. The total extraction efficiency (%) was concentration dependent and ranged between 70.4 and 100.4%. The lower limit of quantification (LLOQ) for all analytes was 10 ng/mL. Repeatability and intermediate precision were ≤9.4 and ≤9.9%, respectively. In order to measure the prevalence of nicotine exposure during the 2009 Ice Hockey World Championships, 72 samples were collected and analyzed after the minimum of 3 months storage period and complete removal of identification means as required by the 2009 International Standards for Laboratories (ISL). Nicotine and/or metabolites were detected in every urine sample, while concentration measurements indicated an exposure within the last 3 days for eight specimens out of ten. Concentrations of nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N′*-oxide and cotinine-*N*-oxide were found to range between 11 and 19,750, 13 and 10,475, 10 and 8217, 11 and 3396, and 13 and 1640 ng/mL, respectively. When proposing conservative concentration limits for nicotine consumption prior and/or during the games (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-*N′*-oxide and cotinine-*N*-oxide), about half of the hockey players were qualified as consumers. These findings significantly support the likelihood of extensive smokeless nicotine consumption. However, since such conclusions can only be hypothesized, the potential use of smokeless tobacco as a doping agent in ice hockey requires further investigation.

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1. Introduction

Nicotine is the principal natural alkaloid present in tobacco leaves. A wide variety of consumption patterns exist, from tobacco smoking, in the form of cigarettes, cigars or pipes, to smokeless tobacco products such as snus, snuff and chewing tobacco. Nicotine replacement therapies also contain this natural compound, as marketed in transdermal patches, nasal sprays, inhalers and gums [1].

Depending on the type of product, concentrations differ to a reasonable extent. On average, a similar content of nicotine is found in cigarette and oral snuff, whereas cigar and chewing tobacco contain only about half of this concentration [1]. Accordingly, levels of nicotine intakes and metabolism pathways vary along these different trends of tobacco consumption. When smoked and inhaled, nicotine is rapidly absorbed in the lungs, reaching the brain via the bloodstream within 20 s [1]. Depending on the pH, there is little to large buccal absorption, which is directly related to the type of product [2,3]. Chewing tobacco and snus are buffered to facilitate absorption of nicotine through oral mucosa. A portion of nicotine is usually swallowed with saliva and well absorbed in the small

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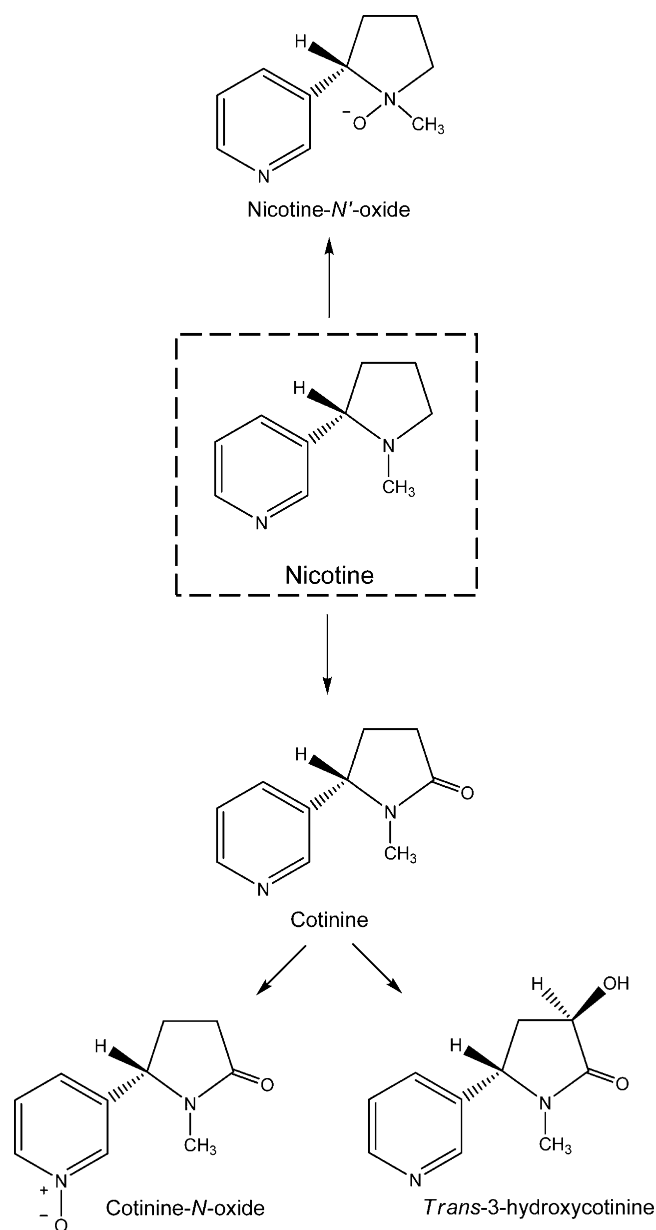

Fig. 1. Simplified metabolic pathway of nicotine [1].

intestine. Concentration in the brain rises at a slower rate than with smoking and levels are declining over a longer period of time. Nicotine is also well absorbed through the skin which is the basis for transdermal delivery that occurs over a long period of time [4].

Nicotine is primarily and extensively metabolized in the liver by *C*-oxidation to cotinine [2,5]. *N*-oxidation also converts nicotine into nicotine-*N*′-oxide and other minor metabolites. Cotinine is further hydroxylated to *trans*-3-hydroxycotinine and also converted to cotinine-*N*-oxide and other minor metabolites by *N*-oxidation (Fig. 1). Simultaneous determination of free urinary nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide account for 8–10, 10–15, 33–40, 4–7 and 2–5% of the total nicotine dose, respectively [5,6].

Due to the relatively short half-life of nicotine in urine (about 2 h), investigating nicotine metabolites which exhibit a longer half-life is a prerequisite to provide relevant information on tobacco consumption [1]. Therefore, an abundant literature on gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods has been published for the determination and quantification of nicotine and selected metabolites in biological fluids, including blood or plasma, urine and saliva [6–14].

LC–MS/MS provides a sensitive and selective approach for comprehensive measurement of free nicotine and its metabolites. However, only very few of these publications bring up a simultaneous, yet steps limited, sample preparation method for the analysis of nicotine and metabolites, in particular nicotine-*N*′-oxide and cotinine-*N*-oxide [6,9,10,12].

Nicotine can act both as a stimulant and a relaxant drug, with predominant effects being an increase in pulse rate and blood pressure, as well as an increase of blood sugar release and the release of epinephrine [4,15]. Positive reinforcing effects also include relaxation, reduced stress, enhanced vigilance, improved cognitive function, mood modulation and lower body weight [3,16].

Thus, when considering nicotine from a doping perspective, consumption in the form of smokeless nicotine products may clearly enhance the performances of sport athletes in various ways as it provides all the effects described above, without the direct health issues usually associated to smoke [17]. Indeed, use of snus, snuff or chewing tobacco has been reported as a growing trend, in particular amongst winter sports such as ice hockey and skiing, but also in other popular sports such as soccer, baseball or basketball and even in fencing or shooting [18–20]. Nevertheless, only old and vague estimates of these consumption patterns have been reported, leading to an extensive underestimate of this potential issue. Nicotine did not appear in the 2009 World Anti-Doping Agency (WADA) Prohibited List or in the 2009 Monitoring Program, a situation which remains unchanged at the present time [21,22]. Thus, investigating nicotine consumption trends amongst professional athletes and developing means to distinguish between consumption of smoke or smokeless nicotine products is of major concern to sport authorities.

Therefore, the project presented in this paper describes an analytical method for the simultaneous determination and quantification of nicotine and its four main metabolites in urine, using liquid–liquid extraction (LLE) followed by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI–MS/MS) in Hydrophilic Interaction Chromatography (HILIC) mode. Apart from a recent publication on nicotine, cotinine and *trans*-3-hydroxycotinine analysis [23], HILIC columns have never been used for such purpose in real biological samples, in particular when including nicotine-*N*′-oxide and cotinine-*N*-oxide. However, this methodology is primarily dedicated to the analysis of polar compounds, such as molecules and related metabolites excreted in urine [24,25]. Owing to the nature of screening procedures for doping agents, a rapid and simple extraction procedure is favoured for comprehensive nicotine consumption study.

This analytical approach has been further applied to the urine samples collected during the 2009 Ice Hockey World Championships held in Switzerland in order to measure the prevalence of nicotine exposure amongst athletes and help to assess the concern associated with nicotine consumption in sport.

2. Experimental

2.1. Reagents and chemicals

(S)-Nicotine (≥99%) and (S)-cotinine (98%) were purchased from Sigma-Aldrich (Buchs, Switzerland), *trans*-3-hydroxycotinine (99.9%), (R/S)-nicotine-*N*′-oxide (98%) and (S)-cotinine-*N*-oxide (98%) were obtained from Toronto Research Chemicals (Toronto, Canada), while (S)-*d*4-nicotine (98.8%), (R/S)-*d*3-cotinine (99%) and *d*3-*trans*-3-hydroxycotinine (98%) were supplied by LGC Promochem (Molsheim, France). Acetonitrile (ACN, ≥99.7%) was

purchased from Biosolve B.V. (Chemie Brunschwig, Basel, Switzerland), propan-2-ol (≥99%) was obtained from BDH Laboratory Supplies (Poole, England), chloroform (≥99.0%) from Acros Organics (Geel, Belgium) and formic acid (∼98%) from Fluka (Buchs, Switzerland). Ammonium formate and disodium hydrogen phosphate were supplied by Sigma-Aldrich (Buchs, Switzerland) and potassium dihydrogen phosphate by VWR International AG (Dietikon, Switzerland). Ultra-pure water was produced by a Milli-Q Gradient A10 water purification system with a Q-Gard® 2 and a QuantumTM EX Ultrapure organex cartridge purchased by Millipore Corp. (Billerica, MA, USA).

2.2. Sample preparation

Urine samples clean-up is based on a method previously published and adapted to our particular needs and matrix [9]. An aliquot of urine (1 mL) was spiked with 10 μl of 10 μg/ml deuterated internal standard (I.S.) solution (*d*4-nicotine, *d*3-cotinine and *d*3-*trans*-3-hydroxycotinine) and diluted with 1 mL phosphate buffer (0.2 M, pH 7.0) prior to vortex mixing. LLE was performed with 2.5 mL chloroform:propan-2-ol (95:5, v/v) for 10 min using a rotator unit. After centrifugation for 5 min at 2500 rpm, the organic layer was evaporated to dryness under a gentle air stream at 50 °C and reconstituted in 1 mL ACN:ammonium formate (10 mM, pH 3.0) (98:2, v/v) prior to LC–MS/MS injection.

2.3. LC conditions

Separation was carried out on a LC–MS/MS system using a Rheos 2000 CPS-LC system pump (Flux Instrument, Basel, Switzerland) and an HTS Pal autosampler (CTC analytics AG, Zwingen, Switzerland). Hydrophilic Interaction Chromatography was performed on a Phenomenex Luna® HILIC column (150 mm × 3.0 mm, 5 μm) (Brechbühler AG, Schlieren, Switzerland) with a guard column SecurityGuard™ HILIC (4 mm × 2.0 mm) (Brechbühler) added to the analytical column. The column temperature and the autosampler tray were set at 30 and 4 °C, respectively. Mobile phase consisted of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min, while partial loop injection volume was 10 μl with a 20 μl loop. The initial mobile phase condition was 98% A for 3 min, which was decreased linearly to 35% and held from 10 to 13 min, then increased back to 98% to re-equilibrate the column from 13.1 to 16 min.

2.4. Linear trap quadrupole-MS parameters

Analyses were performed using a linear ion trap mass spectrometer LTQ-MS (ThermoFinnigan, San Jose, CA, USA) equipped with an atmospheric pressure ionization (API) interface, Ion MAX™, operated in positive ESI mode. MS operating conditions were set as follows: spray voltage = 5.0 kV; heated capillary voltage and temperature of 10 V and 320 °C, respectively; isolation width of 1.5 Da; activation time = 30 ms; activation q of 0.250 and scan time was fixed at 30 ms. Sheath gas, auxiliary gas and sweep gas (nitrogen) were set at 20, 5 and 1.5, respectively.

2.5. Identification criterias

Identification criteria were defined according to the WADA Technical Document addressing this particular topic [26]. The retention time (t_R) tolerance window must be within the range of ±2% between the analyte and the QC of the same batch. Also, concerning MS/MS experiments, three diagnostic ions are required, which may include the precursor ion and with an intensity ≥5% of the most intense diagnostic ion of the MS/MS spectrum. A Signal-to-Noise ratio >3 must be considered and the relative intensity of any of the ions shall not differ by more than 10% (absolute) or 25% (relative) from that of the quality control urine.

2.6. Method validation

2.6.1. Calibration curves

Experiments were conducted following the guidelines on bioanalytical method validation from the US Food and Drug Administration (FDA) and the recommendation of the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop in 2006 [27,28].

A pool of six urine samples from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days was prepared to obtain a negative urine (U_{neg}) for the validation process.

Also, according to the pharmacological effects of nicotine and keeping in mind a doping perspective, only recent consumption of nicotine was of relevant interest. Indeed, trace levels in the 1 ng/mL scale would not provide meaningful quantitative information on the consumption behavior. Thus, in order to ensure statistical significance for further discrimination between recreational, environmental or doping use, selecting a LLOQ of 10 ng/mL through the validation procedure ensured quality quantitative results while maintaining minor bias.

Therefore, the calibration was established over the 10–10,000 ng/mL range for nicotine, cotinine and *trans*-3-hydroxycotinine and 10–5000 ng/mL range for nicotine-*N*′-oxide and cotinine-*N*-oxide. A set of three validation series was achieved, with calibration standards at six concentration levels (k = 6) and validator standards (QC) at four concentration levels (k = 4) prepared in triplicate (n = 3) each time. Calibration curves were built from the peak area ratio of nicotine and metabolites to *d*4-nicotine for nicotine, *d*3-cotinine for cotinine and *d*3-*trans*-3-hydroxycotinine for *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide. Accuracy was expressed as the ratio between the theoretical and the average measured concentration. Repeatability was expressed as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the theoretical value at each concentration level [29]. Intermediate precision was expressed as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration level. An accuracy profile was built for each analyte, combining accuracy and intermediate fidelity variance in the dosing range [30,31]. Data were processed and reported with Xcalibur LCQuan package software from ThermoFinnigan and calculation were performed on Excel 2007 from Microsoft.

The lower limit of quantification (LLOQ) was determined as the lowest QC sample with an acceptable trueness, repeatability and intermediate precision fitting for purpose. Quantitative analysis of nicotine and metabolites in real urine samples was performed using a three-points calibration curve determined and fitted by a linear least-squares regression of the peak area ratios between the analyte and the IS versus concentrations. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank urine preparation.

2.6.2. Selectivity

Influence of endogenous matrix compounds was determined by analyzing urine samples from six individuals certified as negative (<LOD) for nicotine and metabolites. Each sample was extracted in triplicate to highlight the presence of potential interfering matrix compounds within selected tolerance windows.

Accordingly, influence of exogenous xenobiotics was determined by analyzing urine samples from over 250 individuals with

different nicotine consumption habits who reported the use of very various substances appearing on the 2009 Prohibited List and Monitoring Program.

2.6.3. Carry-over

Carry-over was evaluated correspondingly by injecting a blank urine sample subsequently to the analysis of the highest calibrator. This experiment was conducted in triplicate.

2.6.4. Matrix effects

Matrix effects on the ionization response and extraction efficiency were further evaluated along the recommendations published elsewhere [32]. A neat solution was fortified at low, medium and high concentration in the initial mobile phase ACN:ammonium formate 10 mM (pH 3.0) buffer (98:2) (a), while a set of 6 negative urines was also fortified in duplicate prior to extraction (b) and another set of blank urine specimens was extracted and fortified only after (c). By comparing the absolute peak areas of two sets of solutions, matrix effect and extraction efficiency can be evaluated, as reported below (Eqs. (1) and (2)).

$$\text{Matrix effect (ME)} = \frac{c}{a} \quad (1)$$

$$\text{Extraction efficiency (RE)} = \frac{b}{c} \quad (2)$$

2.6.5. Stability

The effect of storage conditions was studied by performing a longitudinal stability assay. Analyte stability was evaluated by monitoring the influence of successive freeze and thaw cycles of QC urine samples at low, medium and high concentrations over a period of 6 months. As real urine samples were stored at −20 °C in a sealed box since their collection, the QCs were handled likewise and defrosted at ambient temperature twice a month for LC–MS/MS analysis. The initial integrated peak area was defined as 100%.

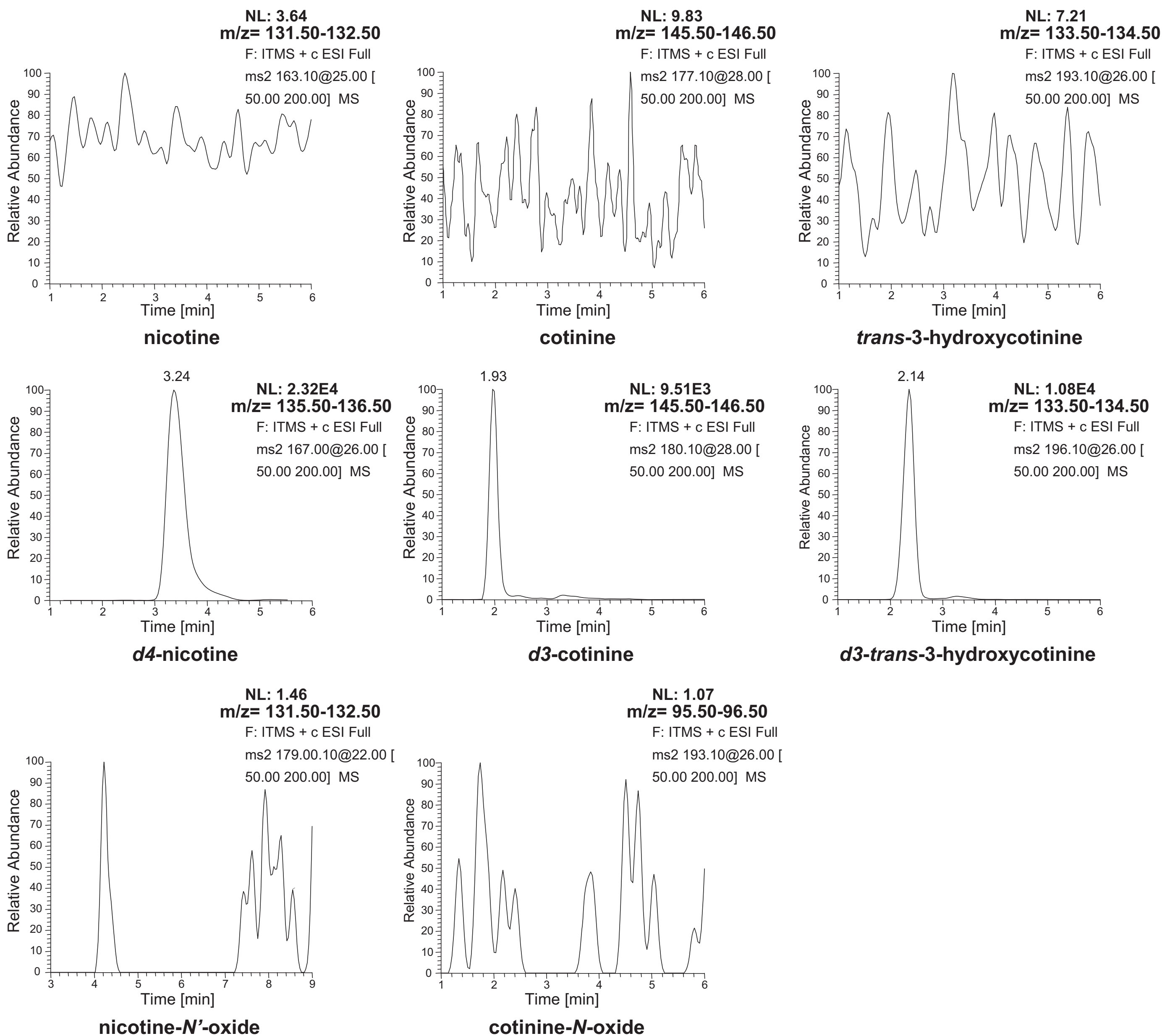

Fig. 2. LC–MS/MS chromatograms of a blank urine as opposed to a urine specimen containing nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N′*-oxide and cotinine-*N*-oxide at a concentration of 10 ng/mL, both with IS spiked at 100 ng/mL. A chromatogram of a urine sample from a hockey player containing the above mentioned metabolites at 1074, 1415, 3739, 2586 and 459 ng/mL, respectively, is also depicted. Quantification ion transitions are in bold.

3. Results and discussion

3.1. Method development

3.1.1. LC–MS/MS analyses

A complete separation of nicotine and metabolites in urine specimens was achieved by hydrophilic interaction chromatography using a gradient of ACN (A) and 10 mM ammonium formate (pH 3.0) buffer (B) with a flow rate set at 0.3 mL/min (Fig. 2). Indeed, HILIC mode allowed to successfully isolate each analyte by providing adequate retention of polar compounds and excellent peak shape. Sensitivity was also optimized since using a mobile phase highly enriched in polar organic solvent ensures an efficient ionization towards the molecules of interest [24]. Likewise, reduced endogenous matrix interferences resulted in very clean chromatograms and a high throughput was obtained due to the feasibility of using a higher flow rate.

Repeatability of the retention times (t_R) was evaluated by calculating mean values variability over the set of three validation series which consisted in 45 extracted samples (Table 1). The RSD obtained were found satisfactory for all the compounds of interest, ranging from 1.8 to 4.1%.

Direct infusion of individual standard solutions, with a flow rate and mobile phase composition corresponding to the elution time from the LC column, allowed optimization of tandem mass spectrometry parameters. Gas streams, spray voltage, heated capillary voltage and temperature, isolation width and compound specific normalized collision energies were manually tuned, resulting in a high sensitivity fragment spectra with a precursor ion response <10% in abundance. SRM transitions, collision energies and retention times for each analyte are provided in Table 1.

3.1.2. LLE

Sample preparation in dope testing favors time and cost efficient procedures which provide satisfactory matrix clean-up and recovery. Thus, the selective extraction protocol for urine samples used in this work was performed with a single LLE. Nicotine and metabolites were neutralized with phosphate buffer at pH 7.0, triggering the extraction with chloroform:propan-2-ol (95:5, v/v). Extraction was followed by evaporation of the organic phase and reconsti-

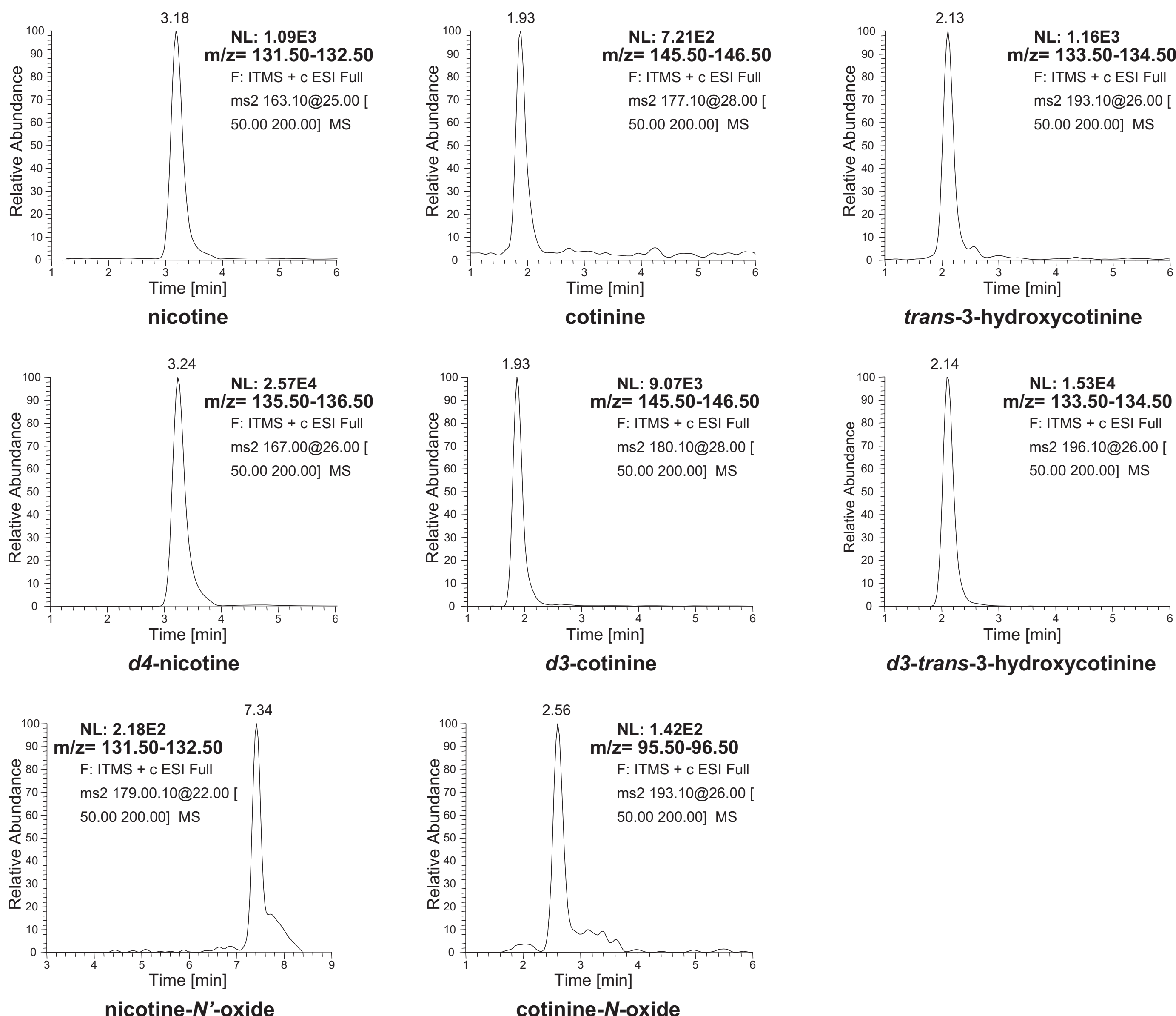

Fig. 2. (*Continued*)

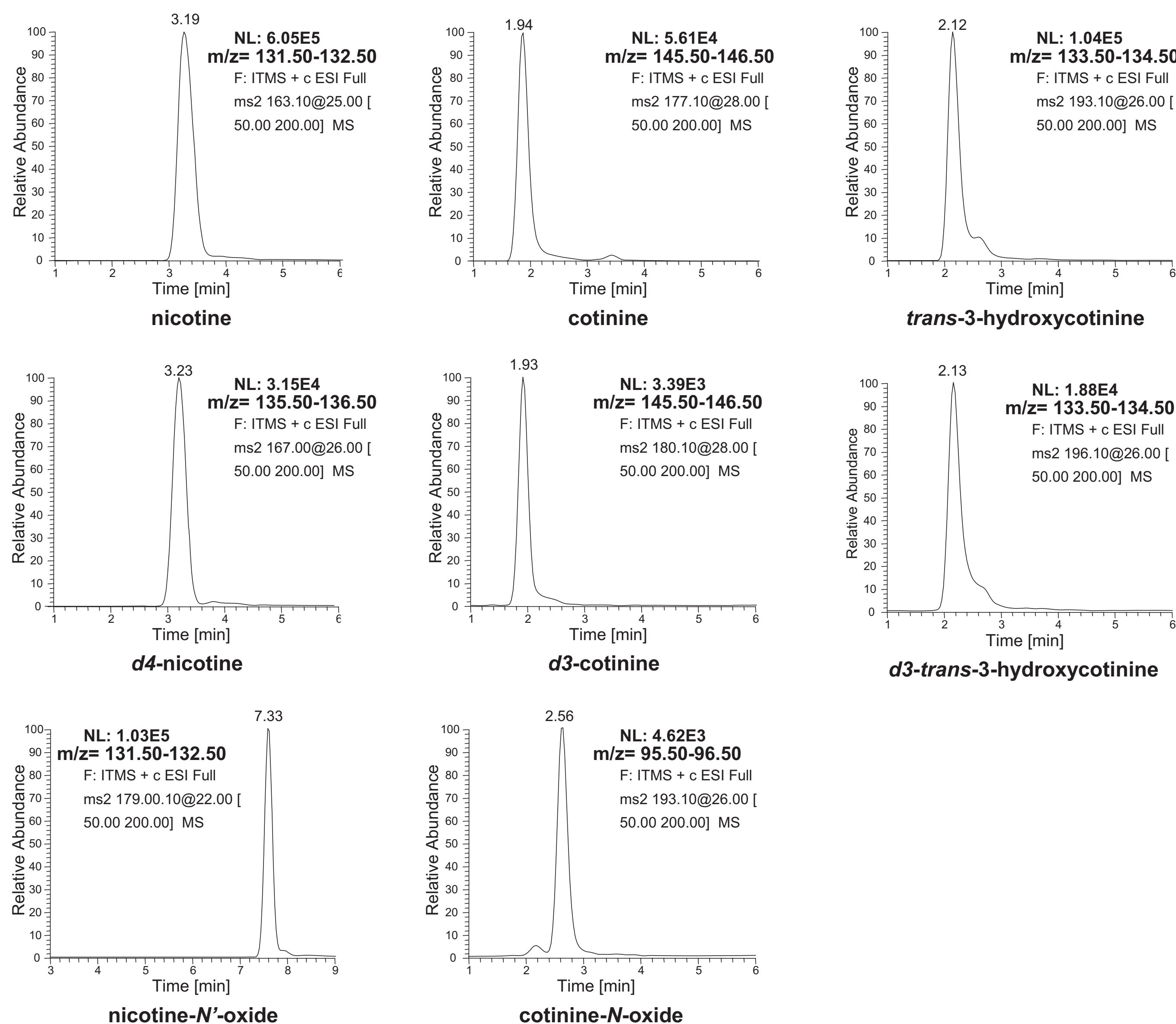

Fig. 2. (*Continued*).

Table 1
SRM parameters and retention times of the analytes.

Analyte	SRM transition (*m/z*)[a]	Collision energy (eV)	t_R (min)
Nicotine	163 → **132**, 120, 106	25	3.18
*d*4-Nicotine	167 → **136**, 124, 110	26	3.24
Cotinine	177 → **146**, 98, 80	28	1.93
*d*3-Cotinine	180 → **146**, 101, 81	28	1.93
trans-3-Hydroxycotinine	193 → **134**, 118, 80	26	2.13
*d*3-*trans*-3-Hydroxycotinine	196 → **134**, 89, 80	26	2.14
Nicotine-*N*′-oxide	179 → **132**, 130, 117	22	7.34
Cotinine-*N*-oxide	193 → 134, **96**, 98	26	2.56

[a] Quantification ion transitions are in bold.

Table 2
Recoveries of nicotine and metabolites at low and high concentrations ($n = 5$).

Analyte	Concentration (ng/mL)			Recovery (%)			RSD (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
Nicotine	10	5000	10,000	95.2	93.4	89.9	3	2	3
Cotinine	10	5000	10,000	99.8	97.6	95.7	9	5	1
trans-3-Hydroxycotinine	10	5000	10,000	70.4	71.6	73.1	5	3	2
Nicotine-*N*′-oxide	10	2500	5000	82.3	83.1	83.2	3	1	0
Cotinine-*N*-oxide	10	2500	5000	76.6	80.5	82.7	0	0	1

Table 3
Assay validation parameters for nicotine and metabolites ($n = 3$).

Analyte	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Nicotine	10	98.4	7.6	8.1
	5000	92.5	6.5	6.6
	10,000	91.8	6.8	6.9
Cotinine	10	105.0	6.4	9.2
	5000	85.5	6.9	7.2
	10,000	109.1	2.7	6.6
trans-3-Hydroxycotinine	10	96.6	7.6	7.8
	5000	89.6	6.0	7.3
	10,000	96.1	5.6	6.2
Nicotine-*N*′-oxide	10	102.4	5.5	5.3
	2500	96.8	9.4	9.9
	5000	94.3	7.7	8.8
Cotinine-*N*-oxide	10	103.9	5.3	5.7
	2500	101.1	5.4	5.9
	5000	89.9	4.6	6.7

tution in the initial mobile phase mixture. This simple, cost and steps-limited methodology provided very clean extracts of urine samples containing nicotine and metabolites. Noteworthy, a batch of 50 items could be prepared within 1 h, allowing a significant workflow of analysis.

RE ranged from 70.4 to 100.4% depending on the analyte, with evidence of good repeatability (RSD < 15%), and showed only slight dependency on the concentration level (Table 2). Indeed, RE for *trans*-3-hydroxycotinine was below what was obtained for the other metabolites. This may result from the pK_a of *trans*-3-hydroxycotinine being much lower compared to the pH of the phosphate buffer.

3.2. Assay validation

3.2.1. Calibration curves

Concentration ranges were initially determined according to expected levels in urine for nicotine and metabolites, while considering both the pharmacological effects of nicotine and a doping perspective which focuses on recent consumption only [33,34]. Thus, in order to ascertain statistical significance for further discrimination between recreational, environmental or doping use, a LLOQ of 10 ng/mL proved to ensure very accurate quantification.

Determination of the best calibration was performed with the evaluation of different curves fitting. Combining accuracy and intermediate fidelity variance allowed building a profile of confidence interval in the dosage range for each target compound [30,31]. According to these accuracy profiles, unweighted linear least-squares regression was found to provide the highest quality results and was chosen for quantification purpose. Due to the linear response, calibration standards were subsequently reduced to LLOQ, medium and ULOQ concentration levels ($k = 3$) and QCs to low, medium and high concentration levels ($k = 3$) with accuracy profiles of comparable quality (Fig. 3). Indeed, accuracy, repeatability and intermediate precision assessments met the guidelines for bioanalytical method validation over the assay range (Table 3). Noteworthy, R^2 corresponding to the initial calibration curve for each compound ($k = 6$) were greater than 0.95, while R^2 with a reduced number of calibrators ($k = 3$) were greater than 0.99. This greatly improved the applicability of this method, allowing a better workflow and simplified calibration.

Therefore, suitability of direct quantification of nicotine and metabolites in urine with this LC–MS/MS method was proven, in particular for nicotine, cotinine and *trans*-3-hydroxycotinine along with nicotine-*N*′-oxide and cotinine-*N*-oxide at concentration ranges of 10–10,000 ng/mL and 10–5000 ng/mL, respectively.

Also, the LOD was found to stand around 500 pg/mL for all compounds.

3.2.2. Selectivity

Selectivity tests towards endogenous matrix compounds were conducted on 6 different urine samples obtained from nicotine-abstinent individuals who had not been exposed to environmental smoke within the last 5 days. After extraction in triplicate followed by LC–MS/MS analysis, no interfering endogenous molecules were observed within selected scan windows since ion identification criteria, including retention times, ion transitions and ion ratios, were not met [26].

Likewise, assessment of potential influence of exogenous xenobiotics was performed on a set of over 250 urine samples collected from individuals of the general population who reported joint exposure of nicotine and different substances present in the 2009 Prohibited List and Monitoring Program. Noteworthy, influence of stimulants most commonly found in urine of hockey players was evaluated, among which caffeine and pseudoephedrine. Again, after extraction and LC–MS/MS analysis, no interfering exogenous xenobiotics were observed within selected scan windows according to the criteria mentioned earlier.

3.2.3. Carry-over

Carry-over was evaluated accordingly, after injection of the highest calibrator (10,000 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 5000 ng/mL for nicotine-*N*′-oxide and cotinine-*N*-oxide), followed by the analysis of a blank urine sample. This procedure was repeated three times successively. None of the target compounds were detected, demonstrating the absence of any carry-over effect.

3.2.4. Matrix effect

ME evaluation by comparison of the signals observed in urine and in the neat solution indicated ion enhancement or suppression depending on the target analyte and concentration. Indeed, nicotine, *d*4-nicotine, nicotine-*N*′-oxide and cotinine-*N*-oxide showed significant ion enhancement at both low, medium and high concentrations, while cotinine, *d*3-cotinine, *trans*-3-hydroxycotinine and *d*3-*trans*-3-hydroxycotinine showed substantial ion suppression at low concentration (data not shown). According to the good repeatability of these assessments (RSD < 15%), along with the satisfactory sensitivity and selectivity of the method, ME influence on the results quality was not significant.

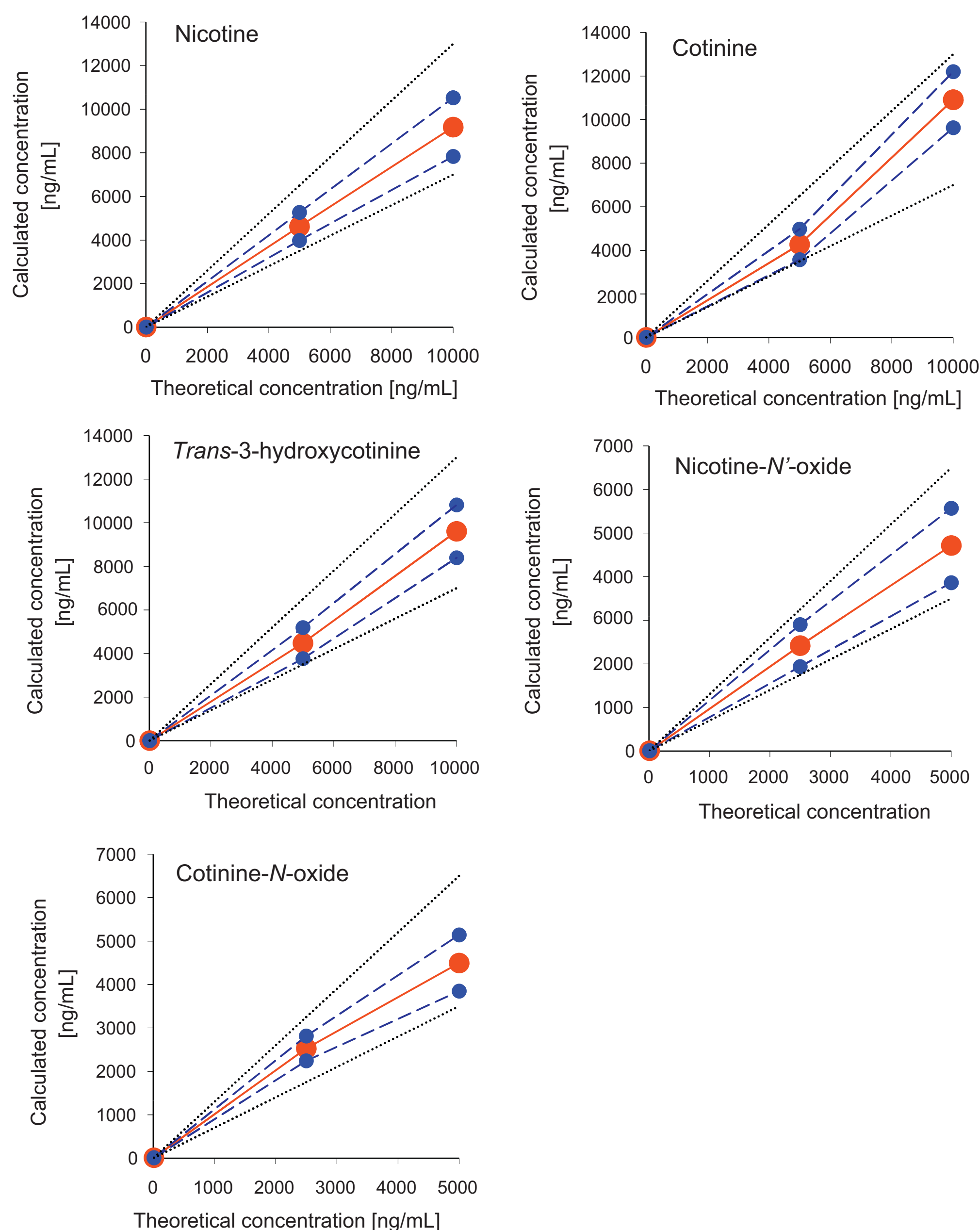

Fig. 3. Absolute accuracy profiles for nicotine and metabolites. The solid line indicates the trueness and the dashed lines represent the accuracy calculated as confidence interval [31]. The dotted lines depict the acceptance limits of ±30%.

3.2.5. Stability

The influence of storage conditions was evaluated by performing a longitudinal stability assay of QC samples every 2 weeks over a period of 6 months. Indeed, these samples experienced freeze and thaw cycles involving successive storage at −20 °C in a complete dark environment and defrost at room temperature, corresponding to storage and analysis conditions during this study.

Referring to the limited variation observed in the peak areas (RSD < 15%), the storage conditions described previously ensured a high stability of all analytes over this particular period of time.

3.3. Application to the IIHF samples

As part of regular doping control protocols during the 2009 IIHF World Championships held in Switzerland, urine samples were collected shortly after every game on two players of each team (n = 72). After approval of the IIHF and Antidoping Switzerland (ADS) and as required by the 2009 International Standards for Laboratories (ISL), article 19 of the World Anti-Doping Code and articles 24–27 of the UNESCO Convention against doping in sport, a minimum storage period of 3 months and complete removal of identification means were ensured prior to use of these samples for research purpose [35–37]. Noteworthy, storage time did not exceed 6 months.

Compounds of interest were quantified in duplicate using a three-point calibration curve together with three urine-based QCs, as described previously. Also, a qualitative value was assigned to metabolites detected in the sub-LLOQ concentration range, namely traces. Concentrations distribution for nicotine and metabolites as quantified in urine specimens are illustrated in Fig. 4. Nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide concentrations ranged between 11 and 19,750, 13 and 10,475, 10 and 8217, 11 and 3396, and 13 and 1640 ng/mL, respectively.

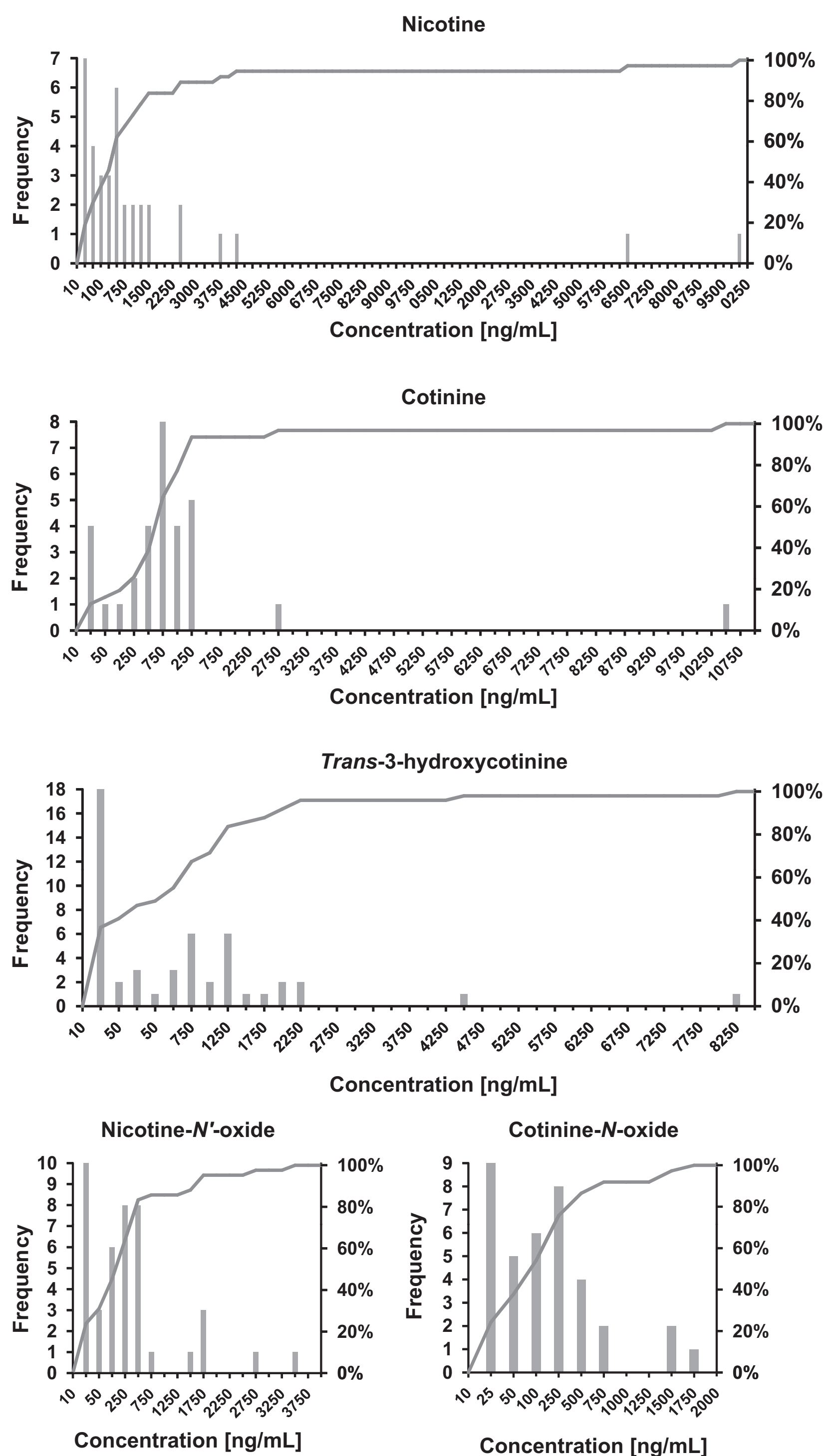

Fig. 4. Concentrations distribution for nicotine and metabolites. The solid line indicates the cumulative percentage.

Traces of nicotine, cotinine, *trans*-3-hydroxycotinine, nicotine-*N*′-oxide and cotinine-*N*-oxide were detected in 87, 91, 94, 97 and 97% of samples, respectively (Table 4). Noteworthy, at least one of the five different metabolites was present in every sample. These findings suggest that every athlete was exposed to nicotine, either environmentally or from active consumption, during the competition period. Such results should be carefully interpreted regarding prevalence studies on smoking in society and on environmental tobacco exposure (ETS) among non-smokers. Indeed, smoking prevalence has been reported by the World Health Organization (WHO) as ranging from 15 to 44% depending on the country participating to the 2009 IIHF World Championships [38]. Also, ETS for a period of at least 1 h per day reached 21% in Switzerland, which hosted the competition [39]. However, both facts may explain only parts of such extensive nicotine exposure, especially when considering that athletes are significantly less likely to smoke or be exposed to smoke than the general population.

Furthermore, above LLOQ levels of the previously mentioned compounds were measured in 51, 43, 68, 58 and 51% of samples, respectively. One of the five different metabolites was detected at

Table 4
Prevalence of IIHF urine samples exposed to nicotine or/and metabolites depending on the concentration range ($n = 72$).

Analyte	Concentration range		
	LOD ≤ x ≤ ULOQ	LLOQ ≤ x ≤ ULOQ	Active exposure
Nicotine	87.5%	51.4%	36.1%
Cotinine	91.7%	43.1%	36.1%
trans-3-Hydroxycotinine	94.4%	68.1%	40.2%
Nicotine-*N*′-oxide	97.2%	58.3%	44.4%
Cotinine-*N*-oxide	97.2%	51.4%	38.8%
Summarized exposure	100.0%	83.3%	52.7%

such concentrations in 82% of samples. These results also indicate that exposure was within the last 3 days previous to the games for approximately 8 ice hockey players out of 10 [40].

Prevalence of nicotine consumption, in the form of smoke or smokeless nicotine, close to or/and during the games was evaluated by hypothesizing conservative concentration limits for active consumption (50 ng/mL for nicotine, cotinine and *trans*-3-hydroxycotinine and 25 ng/mL for nicotine-*N*′-oxide and cotinine-*N*-oxide) [40]. Also, chances of exposure to serious environmental smoke within the few hours prior to games of such importance were excluded. Thus, according to these concentration limits, active nicotine use was highlighted in 36–44% of samples, depending on the target compound (Table 3). Noteworthy, at least one of the five different metabolites was present at such levels in 53% of the urine samples, emphasizing a significant prevalence of nicotine consumption amongst ice hockey players.

Interestingly, two samples presented highly elevated nicotine concentrations exceeding the upper limit of quantification (ULOQ). Such acute exposure to nicotine is hardly achievable for a regular consumer [34,41] thus significantly supporting the likelihood of use for doping purpose. Also, since nicotine half-life is relatively short, consumption close to or/and during the game is the most likely hypothesis. Thus, according to the quantitative measurements performed and the detrimental respiratory effects due to extensive smoking prior to sport practice [42–45], sound evidence on smokeless nicotine use may be hypothesized for these two samples. However, due to the lack of clinical studies addressing metabolic-based distinction between different forms of nicotine consumption, such conclusions could not be extended to other samples. Likewise, specific studies addressing the relationship between nicotine levels and doping are missing, hence the careful assumptions made here.

4. Conclusion

A sensitive and selective HILIC–ESI-MS/MS method for the simultaneous detection and quantification of nicotine and its four principal metabolites in urine was developed and fully validated. The simple and fast sample preparation protocol based on LLE provided a satisfactory matrix clean-up and recovery, while the subsequent use of hydrophilic interaction chromatography allowed to obtain very good separation and peak shape, enhanced sensitivity and high samples throughput.

This analytical procedure was successfully applied to the urine samples collected during the 2009 Ice Hockey World Championships, in order to investigate the prevalence of nicotine consumption amongst athletes. The findings gathered during this work provided strong evidence that nicotine is a very serious trend in ice hockey. Indeed, traces of nicotine or metabolites were found in every urine sample, with concentration levels corresponding to exposure within the last 3 days for approximately eight specimens out of ten. Prevalence of nicotine consumption, in the form of smoke or smokeless nicotine products, before or/and during the games suggested that about half of the ice hockey players were active users. Noteworthy, highly elevated nicotine concentrations were measured in two samples, significantly supporting the likelihood of use of smokeless tobacco for doping purpose.

Assuming that smoking and sport practice at top level are not compatible, these results give a strong indication on the use of smokeless nicotine in ice hockey. Thus, nicotine consumption in ice hockey is a very serious phenomenon which requires further investigation on its use as a potential doping agent.

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