STUDIA UBB CHEMIA, LIX, 1, 2014 (p. 47-61)
(RECOMMENDED CITATION)

# QUANTIFICATION OF NICOTINE AND COTININE IN TEENAGER'S URINE

**ALINA BRATAN[a,b], MANUELA MINCEA[a], IOANA RODICA LUPSA[b], MARILEN GABRIEL PIRTEA[c] AND VASILE OSTAFE[a,c,*]**

**ABSTRACT.** The aim of the study was to develop an Ultrahigh Performance Liquid Chromatography coupled with Mass Spectrometry (UPLC-MS/MS) method for the simultaneous identification and quantification of nicotine and its principal metabolite cotinine in teenager's urine. Sample preparation was performed by liquid–liquid extraction followed by UPLC–MS/MS operated in electrospray positive ionization (ESI) mode with selective reaction monitoring (SRM) data acquisition. In order to measure the prevalence of tobacco consume in adolescents, 150 samples were collected and analyzed. The experiments realized for method validation reveled a linear range between LOQ (2.5 ng•mL$^{-1}$) and 1000 ng•mL$^{-1}$ for both nicotine and cotinine. The accuracy was less than 9 %. Repeatability and intermediate precision were ≤7.6 and ≤8.9%, respectively. Concentrations of nicotine and cotinine in smokers adolescents urine were found to range between from 2.5 to 11.170 ng·mL$^{-1}$ for nicotine and 2.5 to 10.530 ng·mL$^{-1}$ for cotinine, respectively. These findings significantly support the likelihood of extensive nicotine consumption through smoking teenagers in Romania.

***Keywords:*** *teenagers, nicotine, cotinine, urine, UPLC-MS, method validation.*

## INTRODUCTION

Tobacco has been smoked for at least the last three thousand years. What at first appeared to be a trend proved to be a nightmare because nowadays even the manufacturers admit that tobacco cigarettes can seriously damage health and they mention this on their cigarette packages. With all the negative advertising done to tobacco, adults and teenagers continue to smoke.

[a] *West University of Timisoara, Timisoara, Faculty of Chemistry – Biology – Geography, Advanced Environmental Research Laboratories, Oituz 4, Timisoara 300086, Romania*
[b] *National Institute of Public Health-Regional Center of Public Health Timisoara, Babes Victor 16, Timisoara, 300226, Romania*
[c] *West University of Timisoara, Multidisciplinary Research Platform "Nicholas Georgescu - Roengen", Oituz 4, Timisoara 300086, Romania*
[*] *Corresponding author: vostafe@cbg.uvt.ro*

Nicotine (Figure 1a) is the most abundant of the volatile alkaloids in the tobacco leaves [1], being responsible for smoking addiction. By weight, nicotine normally represents about 5 % of a tobacco plant [2]. Nicotine acts on nicotinic cholinergic receptors, affecting most organ systems in the body and is a highly addictive drug [3]. Cigarettes contain 8 to 20 mg of nicotine (depending on the brand), but only approximately 1 mg is actually absorbed in the human body [2]. Nicotine presents a relative short half-life ($t_{1/2}$ = 1-2 hours) [4].

An average of 70–80% of the nicotine absorbed by a smoker is metabolized to cotinine [5]. Measurement of cotinine levels provides a sensitive estimate of tobacco smoke exposure. Cotinine (Figure 1b) is the major degradation product of nicotine metabolism in the liver by C-oxidation [6], and can be determined in various biological matrices, including blood, saliva, urine, and semen [5], even after a few days subsequent to individual exposure on tobacco smoke by determining the half-life in the body [7]. Cotinine levels in the blood are not a good markers of nicotine content, whereas urinary excretion of cotinine represents a good indicator being less influenced by the flow of urine and *p*H [8] even after a few days subsequent to individual exposure on tobacco smoke by determining the half-life in the body [7]. Moreover, in a pilot study hair nicotine/cotinine concentrations were determinate for monitoring exposure to tobacco smoke among infants and adults [9].

In human organism, the half-life of cotinine is much higher (10-20 h) than nicotine, being considered as the main biomarker for assessment of environmental exposure to tobacco smoke (passive smoking) [10]. Although there is not a perfect agreement in the scientific literature about the half-life of nicotine and cotinine, all data agreed that the persistence of cotinine in the body is between 10 to 20 times longer than that of nicotine [4, 7, 11, 12]. Even so, it should be noted that cotinine determination enables monitoring of only a relatively short period (3-4 days) of previous exposure to tobacco smoke.

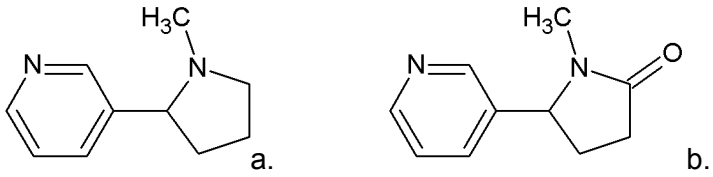


**Figure 1.** Chemical structure of nicotine (a) and cotinine (b).

Exposure to secondhand smoke (SHS) is a significant threat to public health and represents a danger for both the development and health status of children and adolescents[13].

Different analytical methods, including chromatographic techniques interfaced with mass spectrometry, thin layer chromatography, and several immunologically based detection systems [14-19], have been used for determination of nicotine, cotinine and/or other metabolites in urine. Methods combining chromatography and mass spectrometry allow for a Limit of Detection (LOD) of around 1 ng / mL at a cost of approximately $25 per sample, while the enzyme immunoassay (EIA) tests are less expensive, costing about $15 per sample, but less precise with a LOD of 10 ng / mL. Also, due to cross-reactivity with other nicotine metabolites EIA may overestimate cotinine concentrations [20]. However, the chromatographic assays can have the capability of being more specific, particularly when they are interfaced with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) [21].

In Romania, tobacco consumption represents an important public health issue, the statistics showing that more than 33000 people die yearly from smoking. One important reason of high rates of tobacco consumption in Romania is explained by high frequency of smoking among women and youth in general. Growth of this percent was constant in women: 11.3% of women aged over 15 years was reported in 1989, 15.2% in 1994 and 25% in 2000. Also, 21% of the population of Romania was already smoking daily at 15 years of age [22]. These studies were performed with epidemiological methods, based on responses to special questionnaires. In this paper we present a study based on a special questionnaire, but also on a chromatographic analysis of urine of the subjects, in this way providing objective results.

This paper presents an analytical method for the simultaneous determination and quantification of nicotine and its main metabolite, cotinine in urine, using liquid–liquid extraction (LLE) followed by ultrahigh performance liquid chromatography coupled with electrospray ionization - mass spectrometry (UPLC – MS). The method was applied to quantify nicotine and cotinine in urine samples of 150 adolescents from western part of Romania. Such study was not performed in Romania until now.

## RESULTS AND DISCUSSION

### Method Development

A complete separation of nicotine and cotinine in urine specimens was achieved by ultra-performance liquid chromatography using a gradient of acetonitrile (between 10 and 100%) having also 0.1% ammonium formate (buffered at *p*H 5.5), with a flow rate set at 0.4 $mL \cdot min^{-1}$. Although the separation of the two analytes was achieved in less than 0.8 min, the gradient program continued for two min, with the aim to increase the concentration of organic modifier in order to elute from the column the hydrophobic contaminants present in the sample extract.

We successfully isolate each analyte by providing adequate retention of polar compounds and excellent peak shape (Figure 2). Sensitivity was also optimized since using a mobile phase highly enriched in ammonium formate ensured an efficient ionization towards the molecules of interest. 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.

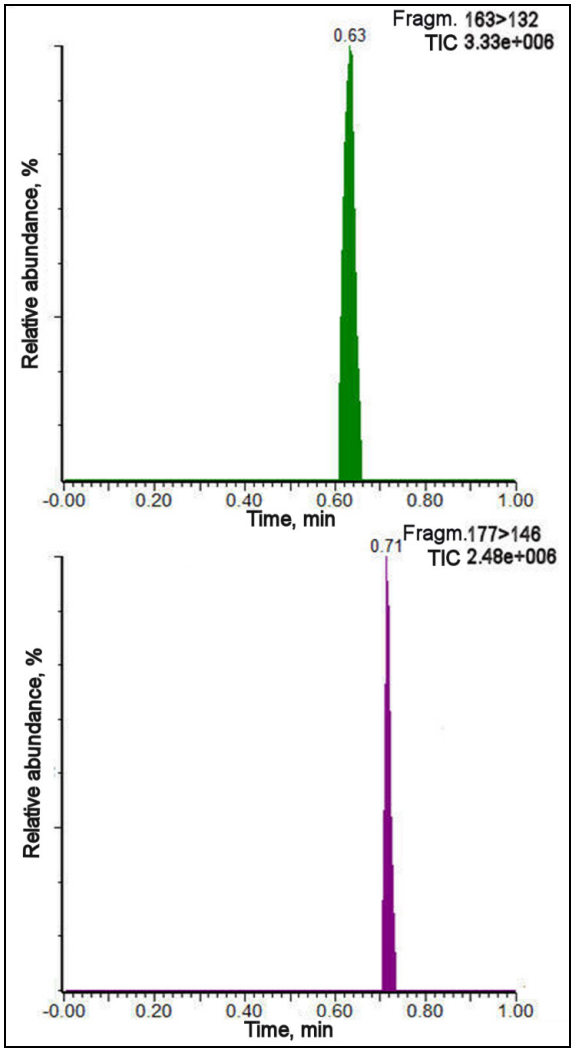


**Figure 2**. Examples of UPLC – MS chromatograms of standard nicotine ($R_t$ 0.63, MRM 163 > 132) and cotinine ($R_t$ 0.71, MRM 177 > 146).

Repeatability of the retention times ($R_t$) was evaluated by calculating mean values variability over the set of three values. The results have proved to be satisfactory for both compounds of interest as repeatability was 0.8% for nicotine and 1.1% for cotinine, respectively. 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 and compound specific normalized collision energies were manually tuned, resulting in a high sensitivity fragment spectra with a precursor ion response <10% in abundance.

### Sample Preparation

The selective extraction protocol for urine samples used in this work was performed with a single LLE. Nicotine and cotinine were extracted from urine with chloroform: propan-2-ol (95:5, v/v), after neutralization with phosphate buffer at *p*H 7.0. Extraction was followed by evaporation of the organic phase and reconstitution in the initial mobile phase mixture. This simple, cost and steps-limited methodology provided very clean extracts of urine samples containing nicotine and cotinine.

### Method Validation

The calibration graph resulted from the analysis of the calibration standard solutions prepared in pooled urine from non-smoking test persons was linear between the quantification limit (2.5 $ng \cdot mL^{-1}$) and 1000 $ng \cdot mL^{-1}$ urine (the highest concentration used for the realization of standard curves) (see Table 1).

**Table 1.** Validation parameters of standard curves for nicotine and cotinine (n = 6) realized in negative urines

| Analyte | Concentration ($ng \cdot mL^{-1}$) | Accuracy (%) | Repeatability (%) | Intermediate precision (%) |
|---|---|---|---|---|
| Nicotine | 10 | 0.91 | 7.6 | 8.9 |
| | 100 | 0.93 | 6.5 | 7.8 |
| | 1000 | 0.97 | 6.1 | 7.1 |
| Cotinine | 10 | 0.92 | 7.1 | 8.7 |
| | 100 | 0.95 | 6.6 | 8.0 |
| | 1000 | 0.97 | 6.2 | 7.4 |

### Accuracy

The loss due to processing was determined to check the accuracy of the method. For this purpose reference standards prepared in water and urine were processed and analyzed. Standards solution prepared in mobile phase with the same cotinine concentrations as the reference standards in their respective matrix were injected in UPLC-MS system without further treatment (n=6). Mean absolute recovery rates for nicotine and cotinine, respectively, of 96.7% and 97.1% (in water) and 93.6% and 94.6% (in pooled urine) were obtained by comparison the direct injection results. This means that 3.3% of nicotine and 2.9% cotinine are lost during the processing and analysis of aqueous analytes samples. In the case of urine samples, the losses due to processing are about 6.4% and 5.4%, respectively.

### Recovery

Trueness was evaluated through recovery studies, fortifying blank urine samples at three levels (10, 100, and 1000 $ng \cdot mL^{-1}$). Each level was replicated six times and the obtained results can be observed in Table 2. Recoveries were higher than 90% for all the analytes and levels assayed.

**Table 2.** Recoveries of nicotine and cotinine (n = 6)

| Analyte | Concentration ($ng \cdot mL^{-1}$) | | | Recovery (%) | | | Relative Standard Deviation (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | L | M | H | L | M | H | L | M | H |
| Nicotine | 0 | 100 | 1000 | 91.3 | 96.8 | 96.9 | 7.3 | 6.2 | 6.3 |
| Cotinine | 0 | 100 | 1000 | 89.6 | 97.6 | 95.1 | 7.9 | 6.5 | 6.6 |

L = low, M = medium, H = high, referring to the level of concentration of the analytes.

### Carry-over

Carry-over was evaluated accordingly, after injection of the highest calibrator (1000 $ng \cdot mL^{-1}$ for nicotine and cotinine), followed by the analysis of a blank urine sample. This procedure was repeated three times successively. Due to the fact that the column was systematically cleaned with 100% acetonitrile, as pointed out in the description of the elution gradient program, none of the target compounds were detected, demonstrating the absence of any carry-over effect.

### Determination of Nicotine and Cotinine in Teenager's Urine Sample

Nicotine and cotinine levels have earlier been used to validate the smoking status of an individual [23, 24]. In order to assess the effects of tobacco use on human health [25, 26], these biomarkers have also been used

in epidemiological studies [27-30], to estimate the passive smoking, and for assessment of the efficacy of quit smoking methods [31]. While studies on nicotine and cotinine levels in cigarette smokers as well as those for passive smoking in other ethnic groups are well documented, for Romanian teenagers there are not reliable data issued by high confidence method, as UPLC-MS.

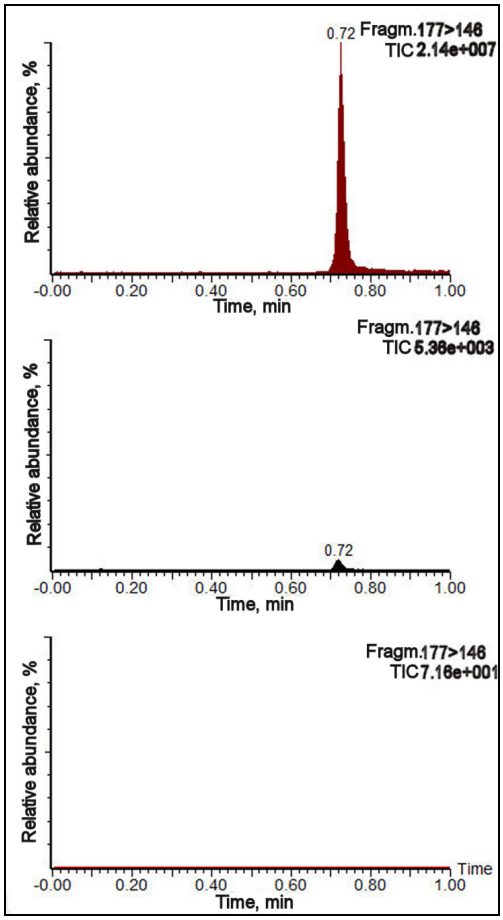


**Figure 3.** Comparison of chromatographic profiles of urine samples collected from a nonsmoker (lower panel), a passive smoker (middle panel) and an active smoker (upper panel). MRM for cotinine 177 >146.

Asking individuals to self-report tobacco use is a cheap method that allowed the collection of detailed information on tobacco use [20]. Self-reports have generally been found to be accurate, but may be prone to recall bias or intentional misreporting [32]. Kandel *et al.* also examined the discrepancies

between self-reported cigarette smoking and salivary cotinine concentration among adolescents. Despite interview procedures designed to emphasize the confidentiality of the interview and to explain to adolescents that they would provide a biological sample to be assayed for the presence of tobacco products, about 42% of the adolescents who reported smoking in the last 3 days had salivary cotinine concentrations below the cut-point, whereas 49% of adolescents with salivary cotinine above the cut-point reported not having smoked within the last 3 days [33]. Among groups for whom smoking is supposed as being undesirable intentional underreporting was observed [34]. In situations where the validity of self-report data is suspect, biomarkers for tobacco exposure provide an objective assess and supply a measure of average or cumulative exposure over a period of time [20].

The concentrations for the urines of the target group ranged from 2.5 (LOQ) to 11.170 $ng \cdot mL^{-1}$ for nicotine and 2.5 (LOQ) to 10.530 $ng \cdot mL^{-1}$ for cotinine. Some chromatographic profiles are presented in Figure 3, as examples of the urine without cotinine, with low level of cotinine (between 10 and 100 $ng \cdot mL^{-1}$) and with high level (more than 100 $ng \cdot mL^{-1}$), respectively.

As we can see from Table 3 the number of teenagers that have declares themselves as active smokers is lower than the number of adolescents that have urine levels of cotinine higher than 100 ng / mL, and can be considered in this category based on an objective criterion. These results are not surprising as at least some of the tested students tend to lie about their smoking status.

**Table 3.** Classification of the subjects according with the cotinine cut-off level found in urines.

| No subjects | AS-d* | AS-a** | PS-d* | PS-a** | NS-d* | NS-a** |
|---|---|---|---|---|---|---|
| 150 | 27 | 31 | 76 | 67 | 47 | 52 |
| % | 18 | 20.66 | 50.67 | 44.67 | 31.33 | 34.67 |
| Cotinine range ($ng \cdot mL^{-1}$) | >100 | | 10-100 | | <10 | |

* d = status declared in the questionnaire,
**a = status assigned after urine analysis
AS = active smokers, PS = passive smokers, NS = non-smokers

Discrepancies were seen also in the situation of passive smokers, as less teenagers were classified in this category based on the UPLC-MS analysis as they have declare in the questionnaires. This lack of concordance can be assigned to the fact that, although these subjects were living in families where the parents are active smokers, the effect of this habit of the adults is not so intense on the state of the children. Quite vice versa was the situation of

non-smokers, where less passive non-smokers than those declared were found by UPLC-MS urine analysis. In this case, we can assume that even these high school students do not live in family with smokers, they spend some time in the presence of smokers.

In all the cases where the UPLC-MS analysis indicated cotinine values higher than 10 ng·mL$^{-1}$, the urine was fortified with cotinine standard in order to confirm the presence of this analyte by the same retention time. An example is presented in Figure 4.

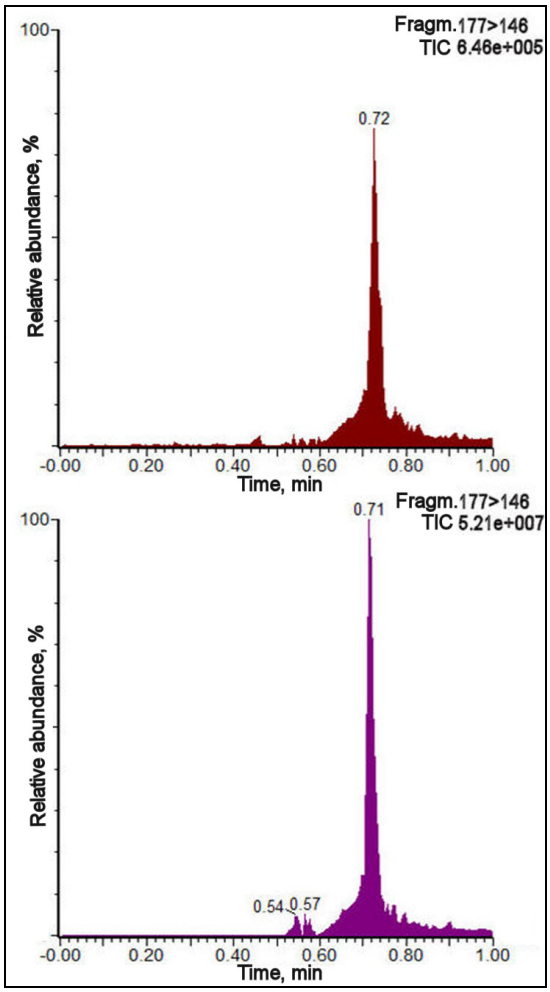


**Figure 4.** Example of chromatographic profiles of a urine sample from an active smoker (upper panel) and the same urine fortified with 100 ng cotinine. MRM for cotinine 177 > 146.

From the 150 samples, 42 have nicotine and cotinine levels below the quantification limit (less than 2.5 $ng \cdot mL^{-1}$ of each compound), *e. g.* 28% of the investigated teenagers were for sure non-smokers. Other 10 adolescents were added to the non-smoker category as the level of cotinine was less than 10 $ng \cdot mL^{-1}$ and they have declared themselves either to be non-smokers living in a non-smokers environment or non-smokers living in smokers families. In total, 34.6% of the subjects were considered as non-smokers, based on the cotinine level in their urine. Based on the UPLC-MS analysis of the urines of the subjects, we have found that 20.6% of the high school students from western part of Romania are active smokers. These results are in a good concordance with other studies [22]. Similar results were obtained when nicotine levels of urine were taken into account. For all the subjects the creatinine levels were in the normal range 5.5 – 11 $ng \cdot mL^{-1}$. This study is the first report of use of an UPLC-MS analytical method to assess the nicotine and cotinine urinary levels of teenagers in Romania.

## CONCLUSIONS

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

The total time to complete this assay is substantially shorter than for other methods that require extensive extractions before assay. The simple extraction into chloroform: propanol mixture combined with a 2 min assay time allowed tens of samples to be analyzed in 8 h working time.

This analytical procedure was successfully applied to the urine samples collected in order to investigate the prevalence of smoking amongst teenagers. The findings gathered during this work provided strong evidence that smoking is a very serious trend in adolescents. Indeed, traces of nicotine and cotinine were found in 72% of urine samples. Prevalence of nicotine consumption, in the form of smoke products, suggested that about 20% of the teenagers were active smokers.

The method was validated according to international guidelines and was applied to quantify the amount of nicotine and cotinine in 150 teenagers, being the first report of such a study performed in Romania.

## EXPERIMENTAL SECTION

### Chemicals and Reagents

(*S*)-Nicotine ((*S*)-3-(1-Methyl-2-pyrrolidinyl)pyridine, Fluka N5511, 1 mg·mL$^{-1}$ in methanol) and (*S*)-cotinine ((*S*)-1-Methyl-5-(3-pyridyl)-2-pyrrolidinone, Fluka C0430, 1 mg·mL$^{-1}$ in methanol) were purchased from Sigma-Aldrich. All other solvents and reagents were of chromatography quality, purchased also from Sigma – Aldrich: ammonium formate (12466 Fluka), formic acid (06440 Fluka), chloroform (CHROMASOLV, 650438 Sigma), 2-propanol (LC-MS CHROMASOLV, 34965 Fluka), acetonitrile (LC-MS CRHOMASOLV, Fluka 14261). HPLC grade water was prepared by SG Ultra Clear 2001-B Water Deionization System (Cole-Parmer) and additionally filtered through syringe filters PTFE 0.22 $\mu$m (Teknokroma, Barcelona, Spain) immediately before use.

The working solutions at concentration level of 1 mg·mL$^{-1}$ of each standard were made in 0.1% ammonium formate in 10% acetonitrile (buffered at *p*H 5.5) and from this, the calibration standards dilutions between 1 mg·mL$^{-1}$ till 0.1 ng·mL$^{-1}$ were freshly prepared before each analytical series.

### Urine Sample Collection Method

All subjects were high school teenagers, from Timisoara, a major city from western part or Romania. The cohort consisted of 150 subjects with ages between 14 to 19 years, from which 27 subjects has declared to be nonsmokers (NS) and to live in an environment where smoking was not allowed, 76 passive smokers (PS) who declared to not smoke but lived with a smoker and 47 declared themselves to be active smokers (AS) who smoked daily at least three cigarettes. All subjects filled out a questionnaire concerning smoking habits. Subjects were instructed on how to collect urine samples when they arose in the morning. Smokers were asked not to have their first cigarette of the day before the samples were collected. Spontaneous urine samples were collected in sealable polyethylene bottles and stored in the deep-freezer at approx. -18 °C until sample processing for nicotine and cotinine determination was carried out. Urine creatinine was measured using standard methodology [35].

### Sample Preparation

Because urine is relatively protein-free a simple liquid-liquid extraction specimen preparation was preferred. An aliquot of urine (2 mL) was diluted with 1 mL phosphate buffer (0.2 M, *p*H 7.0) prior to vortex mixing or with 900 $\mu$L phosphate buffer and with 100 $\mu$L of standards solution (1 $\mu$g·mL$^{-1}$ of each of the analytes). Liquid-liquid extraction (LLE) was performed with 2.5 mL chloroform:propan-2-ol (95:5, v/v) for 10 min using a rotator unit [36].

After centrifugation for 5 min at 2500 rpm, the organic layer was collected and further evaporated to dryness under a gentle nitrogen stream at 50ºC. The extract was reconstituted in 0.5 mL solution of 0.1% ammonium formate in 10% acetonitrile (buffered at *p*H 5.5) and filtered (0.22 *μ*m PTFE) prior to UPLC – MS injection. For samples having very high concentration of nicotine and cotinine, a dilution of the final extract was performed to fit into the calibration range of the standard curves. Calibration is performed using calibration standards which are prepared in pooled urine and are treated in the same manner as the samples to be analyzed.

**Chromatographic Separation and Detection**

Separation was carried out on a Waters Acquity UPLC-MS system (Binary Solvent Manager, Xevo TQD equipped with an electrospray ionization interface). Nicotine and cotinine separation was achieved with a UPLC BEH Phenyl 1.7 *μ*m column (2.1 x 100 mm) using a gradient elution procedure. Mobile phase A consisted in 0.1% ammonium formate in acetonitrile and mobile phase B was 0.1% ammonium formate in 10% acetonitrile. The gradient profile was: 0 – 0.5 min, 100% B; 0.5 – 1 min, 95% B; 1 – 1.5 min, 0% B; 1.5 – 1.9, 0% B; 1.9 – 2 min, 100% B. The column temperature was set at 30°C. The analyses were run at a flow rate of 0.4 $mL \cdot min^{-1}$, and the sample volume injected was 5 μL. The ESI parameters for Xevo TQD MS detector were fixed as follows: capillary voltage at 3 kV, cone voltage at 50 V, source temperature at 120 °C, desolvation temperature at 450 °C, and desolvation gas at 800 $L \cdot h^{-1}$. Nitrogen was used as the desolvation gas, and argon was employed as the collision gas. The detailed MS/MS detection parameters for each analyte are presented in Table 4 and were optimized by direct injection of a 1 $ng \cdot mL^{-1}$ standard solution of each analyte into the detector at a flow rate of 10 $\mu L \cdot min^{-1}$.

**Table 4.** Mass spectrometer parameters for nicotine and cotinine detection

| Analyte | $R_t$ (min) | Precursor ion (m/z) | Quantification ion (m/z) | Collision potential (V) | Confirmation ion (m/z) | Collision potential (V) |
|---|---|---|---|---|---|---|
| Nicotine | 0.63 | 163 | 132 | 25 | 120 | 30 |
| Cotinine | 0.72 | 177 | 146 | 20 | 98 | 35 |

Analyses were carried out in multiple reaction monitoring modes, using two specific transitions for each analyte and the detector was fixed at maximum Extended Dynamic Range with peak mass widths of 2 and 1.5 a.m.u. for the first and third quadruples, respectively. The dwell time for all transitions was 0.01 seconds.

According to the WADA Technical Document addressing to this particular topic identification criteria were defined [37]. The retention time ($R_t$) tolerance window between the analyte and the Quality Control (QC) of the same batch was within the range ± 2%. For MS/MS experiments, three diagnostic ions are required, including the precursor ion and two fragmentation ions.

### Method Validation

Experiments were conducted following the recommendation of the bioanalytical method validation from the US Food and Drug Administration (FDA) and the guidelines on the 3rd American Association of Pharmaceutical Scientists (AAPS)/FDA Bioanalytical Workshop from 2006 [38, 39].

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 negative urine for the validation process.

Accuracy was expressed as the ratio between the theoretical and the average measured concentration.

Repeatability was determined as the relative standard deviation (RSD) of the ratio of the intra-day standard deviation and the theoretical value at each concentration level [40]. Intermediate precision was determined as the RSD of the ratio of the inter-day standard deviation on the theoretical value at each concentration level [41].

The limit of quantification (LOQ) was expressed as the lowest QC sample with a good trueness, repeatability and intermediate precision fitting for purpose.

For recovery experiments of the selected compounds, blank samples (n = 6) fortified with a known amount of analytes before the extraction step (10, 100 and 1000 $ng \cdot mL^{-1}$) were realized.

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.

## ACKNOWLEDGMENTS

This work was supported by the project 464 RoS-NET financed by the EU Instrument for Pre-Accession (IPA) funds, under the framework of the Romania-Republic of Serbia IPA Cross-border Cooperation Programme.

## REFERENCES


1. A.L. Wilson, L.K. Langley, J. Monley, T. Bauer, S. Rottunda, E. McFalls, C. Kovera, J. R. McCarten, *Pharmacol. Biochem. Behav.*, **1995**, *51*, 509-514.
2. L. Vlase, L. Filip, I. Mîndruțău, S. Leucuța, *Stud. Univ. Babes-Bol. Physica*, **2005**, *L(4B)*, 531-535.
3. N.L. Benowitz, *Annu. Rev. Pharmacol. Toxicol.*, **1996**, *36*, 597-613.
4. N.L. Benowitz, P. Jacob, 3rd, *Clin. Pharmacol. Ther.*, **1994**, *56*, 483-493.
5. S.L. Bramer, B. A. Kallungal, *Biomarkers*, **2003**, *8*, 187-203.
6. D. Yildiz, *Toxicon*, **2004**, *43*, 619-632.
7. N.L. Benowitz, S.M. Hall, R.I. Herning, P. Jacob, 3rd, R.T. Jones, A.L. Osman, *N. Engl. J. Med.*, **1983**, *309*, 139-142.
8. D. Behera, R. Uppal, S. Majumdar, *Indian J. Med. Res.*, **2003**, *118*, 129-133.
9. M.N. Tzatzarakis, C.I. Vardavas, I. Terzi, M. Kavalakis, M. Kokkinakis, J. Liesivuori, A. M. Tsatsakis, *Hum. Exp. Toxicol.*, **2012**, *31*, 258-265.
10. T. Welerowicz, K. Śliwka, B. Buszewski, *Chromatographia*, **2007**, *66*, 63-70.
11. N.L. Benowitz, P. Jacob, 3rd, *Br. J. Clin. Pharmacol.*, **2001**, *51*, 53-59.
12. A. Pilotti, *Acta Physiol. Scand. Suppl.*, **1980**, *479*, 13-17.
13. C.I. Vardavas, M.N. Tzatzarakis, M. Plada, A. M. Tsatsakis, A. Papadaki, W. H. Saris, L. A. Moreno, A. G. Kafatos, *Hum. Exp. Toxicol.*, **2010**, *29*, 459-466.
14. P. Dhar, *J. Pharm. Biomed. Anal.*, **2004**, *35*, 155-168.
15. P. Gariti, D.I. Rosenthal, K. Lindell, J. Hansen-Flaschen, J. Shrager, C. Lipkin, A.I. Alterman, L.R. Kaiser, *Cancer Epidemiol. Biomarkers Prev.*, **2002**, *11*, 1123-1125.
16. U.E. Ziegler, J. Kauczok, U.A. Dietz, H.B. Reith, K. Schmidt, *Pharmacology*, **2004**, *72*, 254-259.
17. O.A. Ghosheh, D. Browne, T. Rogers, J. de Leon, L.P. Dwoskin, P.A. Crooks, *J. Pharm. Biomed. Anal.*, **2000**, *23*, 543-549.
18. B.H. Jung, B.C. Chung, S.J. Chung, M.H. Lee, C.K. Shim, *J. Pharm. Biomed. Anal.*, **1999**, *20*, 195-202.
19. C.M. López, A.H. Sassone, M.E. Rodriguez Girault, C.S. Lenzken, E.C. Villaamil Lepori, O.E. Roses, *J. Liq. Chromatogr. R. T.*, **2004**, *27*, 2371-2379.
20. R.M. Kauffman, A.K. Ferketich, D.M. Murray, P.E. Bellair, M.E. Wewers, *Nicotine Tob. Res.*, **2010**, *12*, 582-588.
21. A.N. Hoofnagle, T.J. Laha, P.M. Rainey, S.M. Sadrzadeh, *Am. J. Clin. Pathol.*, **2006**, *126*, 880-887.
22. G. Deaconu, "Study on the use of the pharmacologic therapy with bupropion on tobacco abstinence for smokers with high nicotine dependence", PhD thesis, The University of Medicine and Pharmacy of Craiova, Craiova, Romania, **2010**.
23. G.D. Byrd, K.M. Chang, J.M. Greene, J.D. deBethizy, *Drug Metab. Dispos.*, **1992**, *20*, 192-197.

24. N.T. Lequang, G. Roussel, D. Roche, M.L. Migueres, J. Chretien, O.G. Ekindjian, *Pathol. Biol. (Paris)*, **1994**, *42*, 191-196.
25. I.M. Carey, D.G. Cook, D.P. Strachan, *Epidemiology*, **1999**, *10*, 319-326.
26. F. de Waard, J.M. Kemmeren, L.A. van Ginkel, A.A. Stolker, *Br. J. Cancer*, **1995**, *72*, 784-787.
27. D. Trout, J. Decker, C. Mueller, J.T. Bernert, J. Pirkle, *J. Occup. Environ. Med.*, **1998**, *40*, 270-276.
28. G. Apseloff, H.M. Ashton, H. Friedman, N. Gerber, *Clin. Pharmacol. Ther.*, **1994**, *56*, 460-462.
29. M. Barrueco, R. Cordovilla, M.A. Hernandez-Mezquita, J. M. Gonzalez, J. de Castro, P. Rivas, J. L. Fernandez, F. Gomez, *Med. Clin. (Barc.)*, **1999**, *112*, 251-254.
30. G. Scherer, E. Richter, *Hum. Exp. Toxicol.*, **1997**, *16*, 449-459.
31. G.M. Lawson, R.D. Hurt, L.C. Dale, K.P. Offord, I.T. Croghan, D.R. Schroeder, N.S. Jiang, *J. Clin. Pharmacol.*, **1998**, *38*, 510-516.
32. S. Connor Gorber, S. Schofield-Hurwitz, J. Hardt, G. Levasseur, M. Tremblay, *Nicotine Tob. Res.*, **2009**, *11*, 12-24.
33. D.B. Kandel, C. Schaffran, P.C. Griesler, M.C. Hu, M. Davies, N. Benowitz, *Nicotine Tob. Res.*, **2006**, *8*, 525-537.
34. R.P. Ford, D.M. Tappin, P.J. Schluter, C.J. Wild, *J. Epidemiol. Community Health*, **1997**, *51*, 246-251.
35. M.A. Wall, J. Johnson, P. Jacob, N.L. Benowitz, *Am. J. Public Health*, **1988**, *78*, 699-701.
36. F. Marclay, M. Saugy, *J. Chromatogr. A*, **2010**, *1217*, 7528-7538.
37. WADA, "WADA Technical Document TD2003IDCR. Identification criteria for qualitative assays incorporating chromatography and mass spectrometry", World Anti-Doping Agency (WADA), Montreal (Canada), **2003**.
38. DHHS, FDA, CDER, CVM, "Guidance for Industry: Bioanalytical Method Validation", U.S. Department of Health and Human Services; Food and Drug Administration; Center for Drug Evaluation and Research (CDER); Center for Veterinary Medicine (CVM), Rockville, MD (USA), **2001**.
39. C.T. Viswanathan, S. Bansal, B. Booth, A.J. DeStefano, M.J. Rose, J. Sailstad, V.P. Shah, J.P. Skelly, P.G. Swann, R. Weiner, *Pharm. Res.*, **2007**, *24*, 1962-1973.
40. E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, R. Oprean, S. Rudaz, B. Boulanger, P. Hubert, *J. Chromatogr. A*, **2007**, *1158*, 111-125.
41. S. Rudaz, S. Souverain, C. Schelling, M. Deleers, A. Klomp, A. Norris, T.L. Vu, B. Ariano, J.L. Veuthey, *Anal. Chim. Acta*, **2003**, *492*, 271-282.