ACADEMIA ROMÂNĂ
Revue Roumaine de Chimie
https://www.icf.ro/rrch/

Rev. Roum. Chim.,
2023, *68*(7–8), 357–369
DOI: 10.33224/rrch.2023.68.7-8.05

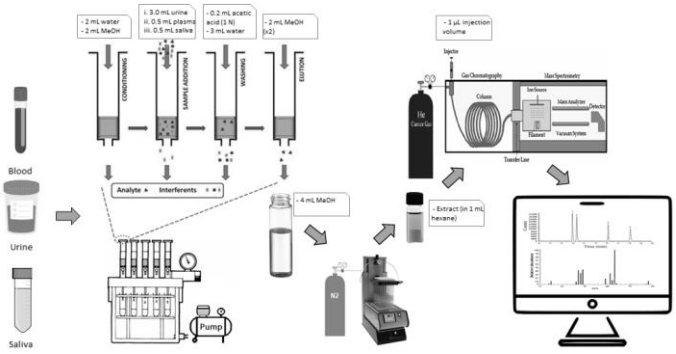
INVESTIGATION OF THE RELATIONSHIP BETWEEN NICOTINE AND COTININE CONCENTRATIONS IN HUMAN BIOLOGICAL SAMPLES BY A GC-MS METHOD**

Emrah DURAL,[a,b,*] Hatice Taslak KOZLUCA,[c,d] Betül İşiner KAYA,[a,e] Görkem MERGEN[a] and Tülin SÖYLEMEZOĞLU[a]

[a] Department of Toxicology, Institute of Forensic Sciences, Ankara University, Ankara 06590, Turkey
[b] Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Cumhuriyet University, Sivas 58140, Turkey
[c] Department of Chest Diseases, Faculty of Medicine, Ankara University, Ankara 06590, Turkey
[d] Chest Diseases Department, Ankara City Hospital, The Ministry of Health of the Republic of Turkey, Ankara 06800, Turkey
[e] Department of Basic Sciences, Faculty of Dentistry, Burdur Mehmet Akif University, Burdur 15030, Turkey

Received September 1, 2022

In this study, a gas chromatography-mass spectrometry method was developed for the determination of nicotine and cotinine wherein human plasma, urine, and saliva. In addition, it was aimed to determine statistically the correlation between nicotine and cotinine levels in urine and saliva samples and nicotine and cotinine levels in blood samples. The limit of quantification was ≤ 0.83 ng/mL and precision were ≤ 4.91 and accuracy (RE%) was between (–4.93) and 4.90. Recovery was detected between 95.4% and 104.7%. The method was employed to determining the nicotine and cotinine concentrations in plasma, saliva, and urine total of 91 samples belong to non-smokers (n = 37) and active smokers (n = 54) who were healthy (n = 65) and COPD patients (n = 27) and the statistical relationship within the nicotine and cotinine values of the samples were investigated. It was found a correlation (r = 0.752, $p \leq 0.01$) between plasma and saliva cotinine levels and estimation equation calculated as $y = 1.56x + 43.24$. Also, the correlation between plasma and urine cotinine levels was found (r = 0.787, $p \leq 0.01$) by the equation that $y = 0.31x + 34.59$. The results show that by accurately determining the amount of cotinine in both saliva and urine, the exposure risks of both active smokers and those exposed to cigarette smoke with the ETS can be estimated.



INTRODUCTION

Nicotine as a primary addictive agent in tobacco plant, a most abundant alkaloid and the primary driver of continued use[1,2] is a naturally derived from the leaves of *Nicotiana tabacum.*[3] People are exposed to nicotine through smoking or inhaling environmental tobacco smoke.[4] In the vast majority of smokers, the primary metabolic pathway is P450 2A6-catalyzed 5′-oxidation, which produces cotinine, the predominant nicotine metabolite in smokers' plasma after the second

* Corresponding author: emrahdural@cumhuriyet.edu.tr; Phone:+90(346)4873892 (office), +90(346)4873938 (Lab)
** Supplementary information on https://www.icf.ro/rrch/ or https://revroum.lew.ro

oxidation even other metabolites have been identified in human urine.[1] Monitoring plasma nicotine levels is effective for assessing nicotine exposure and its pharmacologic effects within a narrow window of exposure; however, it is not ideal for monitoring tobacco exposure over extended periods due to its two hours half-life. In contrast, the determination of blood, salivary or urinary cotinine, the main metabolite of nicotine, is more advantageous as a tobacco biomarker due to it has a longer elimination time and half-life (16–20 hours).[5,6] Although biological materials such as sweat, hair, placenta, meconium, breast milk can be used to determine smoking and second-hand exposure,[7,8] serum, saliva and urine are considered to be very valuable biological materials in smoking-biomonitoring.[9–15]

In Turkey, according to world health organization resport on the global tobacco epidemic focusing to Turkey, it has been reported that 44.1% of men and 19.2% of women (ages +15) are current smokers.[16] In the 1990s and 2000s, studies reporting second-hand smoke exposure became widespread. Although the ban on smoking started in 1996 in the country, it is reported that passive smoking exposure is common, reaching 60% in homes.[17] This widespread smoking behavior observed in the country confirms the increase in passive exposure to a heterogeneous mixture of non-condensed vapors, tar and particulate phase found in cigarette smoke.[14] It is known that both active smoking and passive smoking are harmful to people's health, increasing the risk or severity of cancer, respiratory diseases and cardiovascular diseases.[15] Since the beginning of early 1980s, health risks of passive smoking especially including lung cancer and heart disease have been reported.[14] In biological fluids, nicotine and its metabolite cotinine are used as biochemical markers to predict active smoking behavior, confirm smoking cessation, and evaluate levels and importance of passive smoking exposure.[18,19] In order to predict both active and passive cigarette exposure, economical, practical and powerful analysis techniques are needed for the sensitive, rapid, reliable and simultaneous determination of nicotine and cotinine in biological samples.

In the literature, there are some quantitative determination methods based on radioimmunoassay,[12] thin-layer chromatography (TLC),[20] high-performance thin layer chromatography (HPTLC),[21] high-performance liquid chromatography (HPLC),[10,22,23] capillary gas chromatography,[24] nitrogen-phosphorus detection (NPD).[25] gas chromatography mass selective detection (GC-MS),[12,26] liquid chromatography mass selective detection[27] and tandem mass (LC-MS-MS) techniques have been used[8,28] for determination of nicotine and cotinine in different biological materials as emphasis in above. Although a few methods were established on directly analysis of nicotine and cotinine, their analyses mainly based on after application of an extraction method which were to the specimens. These were liquid-liquid extraction (LLE),[10,14] solid-phase extraction (SPE),[13] solid-phase microextraction (SPME),[8,9] and head-space microextraction (HS-SPME).[29] The SPE method stands out from other extraction methods with its features such as the need for low sample and solvent usage, the observation of strong reproducibility values, the safe application to biological samples with different structures, and the reusability of cartridges.

Although nicotine and cotinine could separate by lots of analytical techniques as mentioned above, gas chromatography (GC) based methods stand out even more because it allows reproducible analyzes of nicotine and cotinine. In addition, the mass detector allows sensitive and reliable detection of nicotine and cones from the extract.

In this investigation, it was aimed to develop a simple, fast, and reliable solid-phase extraction based gas chromatography-mass spectrometry method for determination of nicotine and cotinine levels in human blood, saliva and urine then it was applied to real samples after validation process was accomplished in accordance with the ICH-Q2R1 recommendations.[30] It was intended to generate a correlation equation for estimating blood nicotine and cotinine level by urine and saliva which are not interventional biological materials. On the other hand, with this study, which included non-smokers as a sample group, it was also aimed to determine the nicotine and cotinine levels in biological samples of individuals who were passively exposed to cigarette smoke despite not smoking.

RESULTS AND DISCUSSION

The method showed good chromatographic response without any interference at the retention times of 5.7, 7.1, and 7.5 min for nicotine, diphenylamine and cotinine, respectively. Representative chromatograms belong to real

samples were given in Fig. 1, illustrate the high resolution in ≤ 8 minute which is a short separation time. In the chromatograms obtained from all biological sample applications, no endogenous or exogenous chemical interference was observed that adversely affected the monitorization of the relevant standards, with or without the retention times of nicotine, cotinine, and diphenylamine.

Calibration curves of nicotine and cotinine were drawn by the obtained data with the standard addition method at 12 points (n = 3) between 1 – 5000 ng/mL concentration versus the area of nicotine and cotinine. As individually, each calibration curve was prepared for plasma, saliva and urine. Determination coefficiency (r^2) values calculated between 0.9981 and 0.9999 were given in Table 1. The linearity study was designed to detection of not only low, moderate, and heavy smokers, but also exposures from second-hand (passive) exposure. The nicotine and cotinine levels observed in biological samples clearly showed that the established linearity study was compatible with the aim.

The repeatability study between days was carried out for 5-consecutive days. The data obtained from the accuracy and precision tests, performed in intraday and inter-day with quality control standards established in the blank samples which are prepared in saliva, plasma and urine by standard addition method, showed low RSD% which reflect to precision values ≤ 4.64% and ≤ 4.81% for intraday and interday, respectively. Test results were given in detail in Table 2 and Table 3. Accuracy values (RE%) were between (–3.86) and 4.80 for intraday, (–4.93) and 4.90 for interday (Table 2 and Table 3).

These data showed that when compared with the literature,[14] it is a method with high repeatability that can obtain precise and accurate results in the nicotine and cotinine analyses from 3 samples in the intraday and inter-day reproducibility study (Tables 2 and 3). The obtained intraday and inter-day repeatability values support that the method can be applied safely in real biological samples.

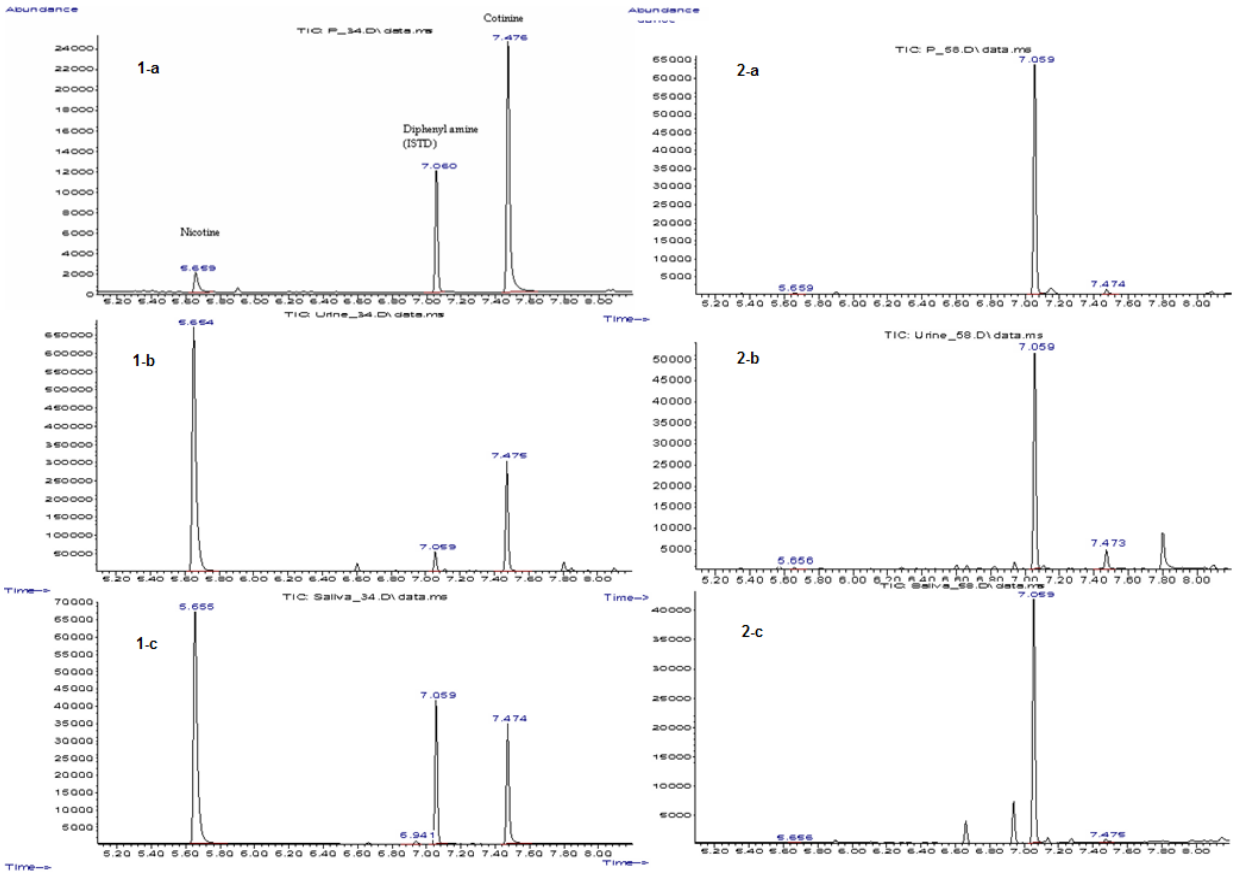


Fig. 1 – Chromatograms showing that nicotine (5.7 min), diphenylamine (7.1 min) and cotinine (7.5 min) analytes and internal standard peaks in plasma, urine, and saliva samples from smokers (1a, 1b, 1c) and non-smokers (2a, 2b, 2c), respectively.

Table 1

Chromatographic characteristics and system suitability parameters of the developed method

Analyte	Matrix	Determination ions (m/z)	Retention time (t_R, min)	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)	Calibration equation	Correlation coefficient (r^2)	Reteniton factor (k')	Theoretical plate number (N)	Separation factor (α)	Resolution (R_s)
Nicotine	Plasma	84 133 161	5.7	0.12	0.36	1 – 5000	$y = 2.5904x – 0.3577$	0.9982	2.8	51984	1.3	9.7
Cotinine	Plasma	98 119 176	7.5	0.23	0.77	1 – 5000	$y = 2.1013x – 0.06166$	0.9983	3.9	21904	1.1	3.0
Nicotine	Saliva	84 133 161	5.7	0.25	0.83	1 – 5000	$y = 0.7304x + 0.0239$	0.9999	2.8	36100	1.3	8.1
Cotinine	Saliva	98 119 176	7.5	0.22	0.73	1 – 5000	$y = 0.5298x – 0.067$	0.9987	3.9	14018	1.1	2.4
Nicotine	Urine	84 133 161	5.7	0.07	0.21	1 – 5000	$y = 0.6351x – 0.0219$	0.9998	2.8	81225	1.3	12.1
Cotinine	Urine	98 119 176	7.5	0.11	0.33	1 – 5000	$y = 0.476x – 0.0838$	0.9981	3.9	38940	1.1	3.9

Abbreviations and backround of equations: retention factor (k') = ; Theoretical plate number (N) = 16^2 ; resolution (R_s) = ; specificity factor (α) = : t_R: retention time of the analyte peak ; t_0: retention time of mobile phase peak; W_t: peak width; LOD: Limit of detection; N: theoretical plate number; r^2: correlation coefficient; α: separation factor; R_s: resolution.

Table 2

Confidence parameters of the method including intraday and interday precision and accuracy values associated with nicotine analysis and recovery data

Matrix	Nicotine Conc. (ng/mL)	Intraday				Inter-day	Average recovery (%)			
		No. Obs.	Observed concentration, $\bar{x}$±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Observed concentration, $\bar{x}$±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	
Plasma	50	5	48.77 ± 2.05	4.20	-2.46	5	48.16 ± 2.21	4.59	-3.68	95.67
	200	5	208.51 ± 7.06	3.39	4.26	5	209.02 ±5.29	4.51	2.53	95.41
	1000	5	991.51 ± 20.98	2.11	-0.85	5	961.47 ± 30.94	3.22	-3.85	104.50
Saliva	50	5	48.56 ± 1.88	3.87	-2.88	5	48.82 ± 2.21	4.53	-2.36	102.43
	200	5	192.29 ± 7.93	4.12	-3.86	5	190.14 ± 8.85	4.66	-4.93	103.88
	1000	5	1040.09 ± 34.51	3.32	4.01	5	1031.57 ± 36.24	3.51	3.16	97.24
Urine	50	5	51.93 ± 2.49	3.86	4.80	5	52.32 ± 2.25	4.30	4.64	96.36
	200	5	192.82 ± 8.30	4.31	-3.59	5	191.63 ± 7.12	3.72	-4.19	101.12
	1000	5	1029.69 ± 34.43	3.34	2.97	5	1036.00 ± 23.14	2.23	3.60	97.42

Note: Nicotine and cotinine as the analytic agents and diphenylamine used as the internal standard which was employed in the study. $\bar{x}$ was used to symbolizes the average value. Standard deviation was abbreviated as SD and relative standard deviation was abbreviated as RSD% calculated with the formula. Accuracy (RE%) of the method that calculated as.

Table 3

Confidence parameters of the method including intraday and interday precision and accuracy values associated with cotinine analysis and recovery data

Matrix	Cotinine Conc. (ng/mL)	Intraday				Inter-day	Average recovery (%)			
		No. Obs.	Observed concentration, $\bar{x}$±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Observed concentration, $\bar{x}$±SD (ng/mL)	Precision (RSD%)	Accuracy (RE%)	
Plasma	50	5	52.22 ± 2.25	4.31	4.44	5	52.04 ± 2.48	4.77	4.08	98.33
	200	5	193.37 ± 5.39	2.79	-3.32	5	196.41 ±6.11	3.11	-1.80	95.39
	1000	5	1036.12 ± 27.65	2.67	3.61	5	1032.91 ± 28.18	2.73	3.29	96.26
Saliva	50	5	51.96 ± 2.29	4.41	3.92	5	52.10 ± 2.56	4.91	4.20	103.19
	200	5	192.42 ± 6.52	3.39	-3.79	5	190.37 ± 6.92	3.63	-4.82	104.37
	1000	5	973.25 ± 24.12	2.48	-2.68	5	950.81 ± 31.73	3.38	-4.91	96.81
Urine	50	5	48.29 ± 2.26	4.68	-3.42	5	48.84 ± 2.35	4.81	-2.32	104.73
	200	5	206.52 ± 9.59	4.64	3.26	5	208.74 ± 6.78	3.25	4.37	97.67
	1000	5	1041.96 ± 42.26	4.06	4.20	5	1049.00 ± 45.63	4.30	4.90	104.09

The extraction yield values for the 3-replicates recovery study for nicotine and cotinine concentrations of 50, 200 and 1000 ng/mL were in the range of 94.36% to 106.88% (Tables 2 and 3). These observed excellent values obtained from the extraction method developed and optimized seemed that analytes, matrix (untreated plasma, saliva and urine) and method (solid-phase) have perfect compatibility between them. It is thought that it will make a significant contribution to the literature on nicotine and cotinine analyses since the recovery value observed is 100.10% on average. The strong correlation between the method-recovery test results and the reproducibility results is considered as a result of the high extraction efficiency at different concentration points of nicotine and cotinine (50, 200, 1000 ng/mL). Raw data used in the calculation of the recovery was detailed in Tables 2 and 3.

Results from method validation data

The linearity study of our research method was calibrated in a wide range of concentrations to include nicotine and cotinine levels in biological samples. In our study, a correlation coefficient equal and higher than 0.998 was determined from the linearity results obtained for both analytes. In the literature, the number of studies using a very wide calibration range such as 1–5000 ng/mL for the detection of nicotine and cotinine in biological samples, as in this study, is limited. As a single example, Shin *et al*. (2002) used a concentration range of 1 to 5000 ng/mL for linearity study in the analysis method, based on GC-MS determination, they developed for the analysis of nicotine and cotinine from urine, plasma and saliva samples.[14] The correlation coefficency (r^2) value determined for the analysis of both analytes was 0.998. In another study, applied by Abu-Qare *et al*. (2001) an HPLC-based investigation, was performed in the range of 200 ng/mL to 2000 ng/mL.[13] However, no information is given about the correlation coefficient data obtained for both nicotine and cotinine in this research. In a scientific investigation that accomplished by Karaconji *et al*. (2007), linearity was established between 1–500 ng/mL concentrations both nicotine and cotinine.[29] Finally, Ramdzan *et al*. (2016), it is seen that the analysis method is established between 5 and 500 ng/mL for cotinine.[11] When the results of the analyzes we performed with real biological samples in this study are examined, it shows that the concentration range in which the method is linear fully meets the values required in the research.

In this study, the accuracy value was determined in the range of (–4.93) to 4.80 and (–4.91) to 4.90 (RE%) for nicotine and cotinine in intraday and interday measurements, respectively. According to study of Shin *et al*. (2002) accuracy values of the reproducibility study were calculated according to the results given in the paper.[14] RE% values for cotinine test were found as ranging from –61.48% to 5%. In nicotine administration, the RE% value was observed to be between (–10)% and 5%.[14] Although the RSD% value increased up to 16.2% in intraday and interday analyzes of the precision study data, performed by hydrophilic interaction liquid chromatography-based analysis, Ramdzan *et al*. (2016), the accuracy of the method was found to be between 0.2 and (–3.15) %RE during and between days.[11] In Abu-Qare *et al*. (2001) study, based on an HPLC-UV determination which established in rat urine and plasma, accuracy values were observed between 2.6 and 3.6 (RE%) for nicotine and 2.4 – 3.2 (RE%) for cotinine.[13] The results showed that the accuracy value determined for nicotine and cotinine in our study was found by Shin *et al*. (2002) clearly showed that it is much stronger than the value found in their study.

The precision values were determined as ≤ 4.91 for both analyte measurement values within and between days. According to Shin *et al*. (2002) found the RSD% value of the precision study for nicotine and cotinine below 5% in all samples.[14] However, in this study, the %RSD value was found below 4.92% both nicotine and cotinine analyses, as the details were given in Tables 2 and 3. When the data obtained from the method validated according to the ICH Q2R1 guideline were considered holistically, the results that were in harmony with each other and this observed precision value were of a quality that could ensure the successful conclusion of the analytical study.

The LOD values determined in this paper for nicotine and cotinine analysis in urine were 0.07 and 0.11 ng/mL, respectively. The LOD values for nicotine and cotinine in the plasma samples were 0.12 and 0.23 ng/mL, respectively. Lastly, the LOD values determined for nicotine and cotinine concentrations in saliva samples were 0.25 and 0.22 ng/mL, respectively (Table I). Shin *et al*. (2002) found the LOD to be 0.2 ng/mL for nicotine and

cotinine values in urine, and 1.0 ng/mL for plasma and urine samples.[14] We have got the lower LOD values in our study for these sample types. These results show 2 times higher LOD for urine nicotine and cotinine, and at least 4 times higher LOD for plasma nicotine and cotinine when compared to our own study. Ramdzan *et al*. (2016) found the LOD value in their study for cotinine analysis in saliva to be 1.5 ng/mL in their research.[11] This value was nearly 7 time higher compare to this suggested study. The robustness of the sensitivity data of our study for all biological samples was particularly useful in detecting nicotine and cotinine levels due to environmental cigarette exposure. The mean values of nicotine and cotinine detected in saliva samples of 37 non-smoking volunteers, respectively, were 4.93 ng/mL ± 10.63, (mean ± SD) and 7.11 ng/mL ± 17.20, indicating the importance of the sensitivity data we obtained. Similarly, in the same group, plasma nicotine and cotinine values were 5.46 ng/mL ± 4.85 and 15.64 ng/mL ± 41.47, respectively, and nicotine and cotinine values detected in urine samples were 67.24 ng/mL ± 390.62 and 22.66 ng/mL ± 87.88, respectively (Supplementary Data II).

In this study, the recovery values of the method developed using the solid-phase extraction technique were found to be between 94.36 and 106.88 % for nicotine, and between 95.39 and 106.73 % for cotinine analysis. The analysis method we developed is based on 3 mL urine sample and 0.5 mL saliva and plasma sample for nicotine and cotinine analysis. Shin *et al*. (2002) used 5 mL urine and 0.5 mL saliva-plasma sample as biological samples. The extraction method developed in our study is based on the solid-phase technique and requires very little solvent in practice. However, Shin *et al*. in the liquid-liquid extraction application developed, a significant amount of a strong organic solvent called, diethyl ether. However, in our study, a total of 4 mL of methyl alcohol with high polarity was used for elution purposes. In addition, the solid phase extraction technique is automated, allowing more than one sample to be prepared at the same time. Ease of application to complex matrices, being an environmentally friendly technique and the high extraction yield power obtained are some of the reasons why it is a more preferred technique today. According to Shin *et al*. (2002) the recovery value of the study was between 88–98% for nicotine and 94–99% for cotinine. In our offered method, the average nicotine recovery values were determined 98.53%, 101.18% and 98.3%, for plasma, saliva and urine speciments, respectively. Likewise, the average cotinine recovery values were determined 96.66%, 101.46% and 102.16%, for plasma, saliva and urine speciments, respectively. The solid-phase extraction application method was simple, practical and fast and offered the opportunity to apply to a large number of biological samples at the same time. At the same time, considering the type and volume of the organic solvent used in the extraction application, it was taken into account that the possible harm of the method to the environment should be kept to a minimum.

Data obtained from selectivity, linearity, accuracy, precision, sensitivity, and recovery tests clearly showed that our proposed method, validated in accordance with the ICH-Q2R1 guideline, is sensitive, reliable, and reproducible for different concentration levels of nicotine and cotinine in blood, urine, and saliva. With these results obtained from validation tests, the method developed to determine nicotine and cotinine concentrations in blood, urine and saliva samples could be safely applied to real biological samples collected from 91 volunteers.

Relationship between nicotine and cotinine levels of plasma, urine and saliva samples

When the nicotine levels of 91 biological samples were examined, the correlation between the its values of plasma and urine samples was $p < 0.01$, $r = 0.673$; in the relationship between the nicotine values of plasma and saliva samples, $p < 0.01$, $r = 0.723$; It was determined that there was a statistical significance and correlation of $p < 0.01$, $r = 0.815$ between the cotinine values of urine and saliva samples (Fig. 3, Supplementary data-I, Supplementary data-II).

The statistical significance value determined here was $p < 0.01$ for all three samples. The strongest relationship appears to be between urine and saliva samples, followed by plasma saliva and plasma urine, respectively.

When the relationship between the amount of cotinine contained in the same samples was examined, $p < 0.01$, $r = 0.787$; between plasma and saliva $p < 0.01$, $r = 0.752$; It was determined that there was a statistically significant relationship and correlation between urine and saliva with $p < 0.01$, $r = 0.832$ (Fig. 2, Supplementary Data I, Supplementary Data II).

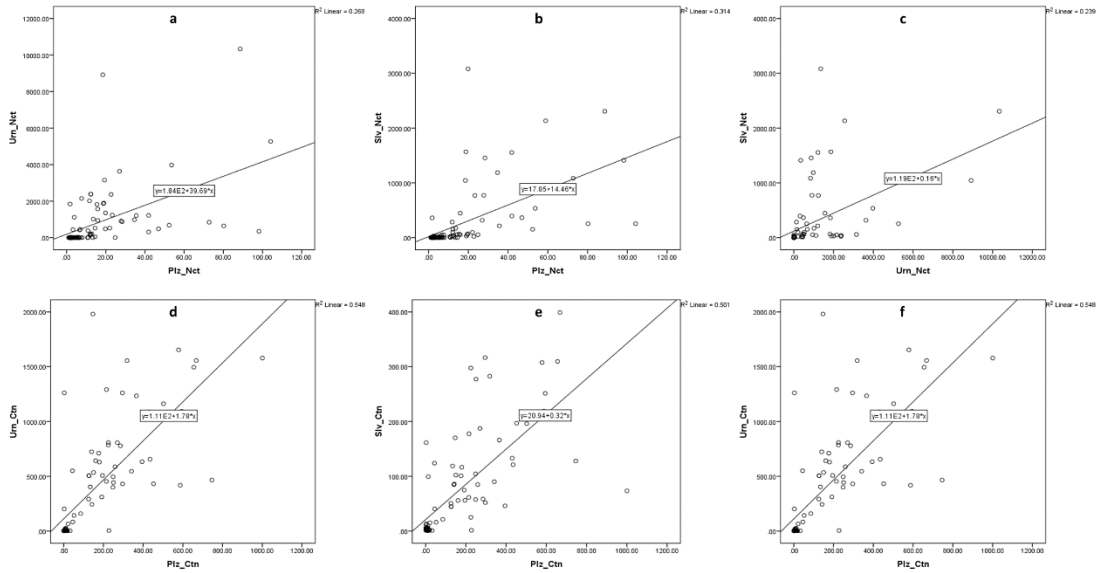


Fig. 2 – Scatter graphs that are showing the correlations of plasma-urine nicotine (a), plasma-saliva nicotine (b), urine-saliva nicotine (c), plasma-urine cotinine (d), plasma-salivary cotinine (e), urine-salivary cotinine (f) concentrations in samples belong to 91 volunteers.

A similar relationship was observed in the relationship between cotinine and biological samples, and the statistical significance level was $p < 0.01$ for all samples. It is seen that the strongest relationship is between urine and saliva samples, but unlike plasma, it is followed by plasma urine and plasma saliva, respectively. It was determined that the mean correlation value of nicotine in biological samples was 0.737, and the mean correlation value of cotinine among biological samples was 0.790. In general, correlation levels of cotinine in biological samples were found to be higher than nicotine.

The relationship between nicotine and cotinine levels

Among the nicotine and cotinine values detected in plasma samples, $p<0.01$, $r = 0.862$; among urine samples $p < 0.01$, $r = 0.916$; Significance and correlation were observed among saliva samples with $p < 0.01$, $r = 0.902$, respectively (Fig. 3). It was observed that the strongest relationship between nicotine and cotinine in biological samples was detected in urine samples.

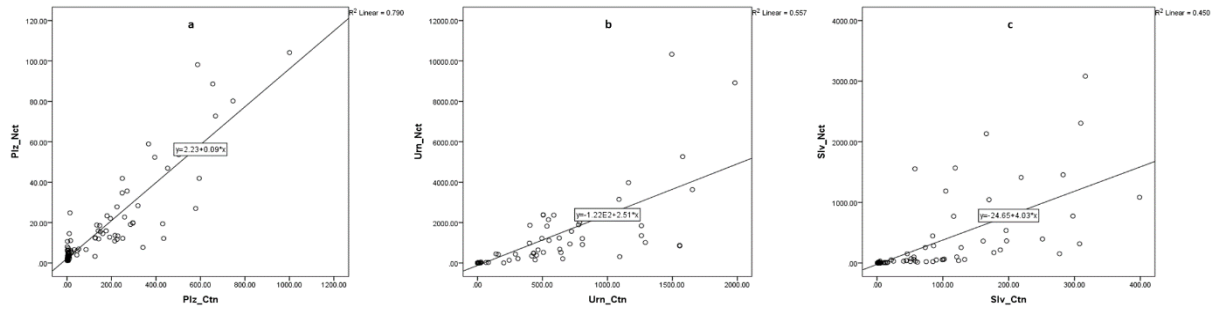


Fig. 3 – Graphs showing that the relationship between nicotine-cotinine concentrations wherein plasma, urine, and saliva samples of 91 volunteers.

Environmental tobacco smoking observation

In our study, nicotine and cotinine concentrations were determined in plasma, urine and saliva samples of 37 non-smoker volunteers. The mean plasma cotinine value was 15.64 ng/mL ± 41.47 (mean ± SD), urine cotinine value was 22.66 ± 87.88, and salivary cotinine value was 7.11 ± 17.20. The study clearly demonstrated the risk of environmental exposure to cigarettes.

EXPERIMENTAL

Chemicals and reagents

Nicotine (98%), cotinine (99%) and diphenylamine (≥ 99%) (Fig. 4) standard chemicals were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol, n-hexane and acetonitrile (gas chromatography grade) and sodium hydroxide, acetic acid, sodium sulfate (analytic grade) were purchased from Merck (Darmstadt, Germany). The ultrapure-water supplied from the Human Corporation UP 900 Scholar-UV model (Seoul, South Korea) water prufiying system that conductivity and electrical resistance were ≤ 0.055 µS/cm (25 °C) and ≤ 18.2 mΩ·cm, respectively. Ultrapure helium gas (≥ 99.9% purity), used as the mobile phase, was supplied from Oksan Inc. (Ankara, Turkey). The Sep-Pac® Vac 3 cc (500 mg) solid-phase C18 cartridge was purchased from Waters ™ (Dublin, Ireland). C18 absorbent particle size is 55–105 µm. Sorbent subsrate is silica, and its pore size is 125Å. Silica-based octadecyl bonded phase has strong hydrophobicity. Typical applications include drugs and their metabolites in serum, plasma or urine, desalination of peptides, trace organics in environmental water samples, organic acids in beverages, as well as to adsorb even weakly hydrophobic analytes in aqueous solutions. A Chromabond solid-phase extraction apparatus was used in the sample treatment step (Dueren, Germany). Sample concentrator (Techne, Essex, UK), combined with nitrogen (99.9% purity), was used in the evaporation.

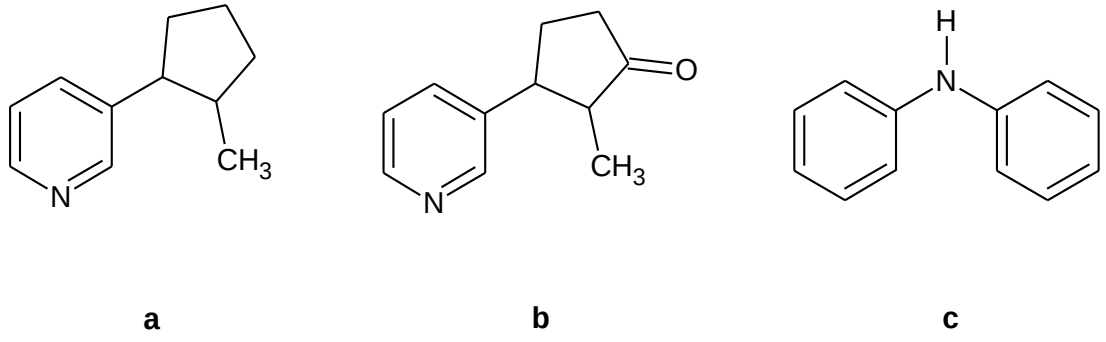


Fig. 4 – Chemical structures of nicotine (a), cotinine (b) which were used as analytes in the study and, diphenylamine (c) that employed as an internal standard.

Instrumentation

An Agilent Hewlett-Packard (HP) GC 6890 (G1530A) series chromatographic system equipped with an automatic liquid sampler (7683B) and mass selective detector (5973) supported a turbo-pump was used in the chromatographic analysis. Analytical separation was accomplished with a DB-5MS (Santa Clara, USA) column 30 m × 0.25 mm, 0.25 µm film-thickness (122–5532 model). Sample prepared with dissolving in n-hexane was injected to the system in splitless mode. Sample injection volume was 1 µL. Helium was constantly applied to the GC-MS system with 1.5 mL/min (14.5 psi pressure) during the analysis.

The optimized oven program was implemented was as follows: initially, the column was held up for 1 min at 80 °C after the sample was injected into the system. Then, the oven temperature was steadily increased to 260 °C with a 25 °C/min ramp. Finally, the column temperature was raised to 300 °C with the 20 °C/min ramp in post-run and it was held at this degree for 4 mins. The interface part was kept at 300 °C. Run time was 10.2, total analysis time was 14.2 min.

Selected ion monitoring (SIM) mode was employed to the quantitation of nicotine and cotinine. The solvent delayed time was 5 min. Electron voltage was 1953 rel. Mass spectroscopy detector source was operated in the electron impact (EI) mode at 70 eV, 230 °C and the mass spectroscopy quadrupole temperature was 150 °C. The major ion peaks of nicotine and cotinine were determined by the scan mode of mass detector between 50–450 amu. Quantitation of analytes were determined with these ions which are 84, 133 and 161 (*m*/*z*) for nicotine; 98, 119 and 176 (*m*/*z*) for cotinine; and 77, 168, and 169 (*m*/*z*) for diphenylamine that used as an internal standard. Quantitation ions were m/z 84 for nicotine; *m*/*z* = 98 for cotinine; *m*/*z* = 169 for internal standard. Dwell time was 100 ms for all ions. The retention times of nicotine, cotinine, and diphenylamine were 5.7, 7.1, and 7.5 min, respectively.

In order to be used as an internal standard in the study, chemicals named quinolone, 1,4-butanediol, fluorobenzene, 5-Aminoquinoline and diphenylamine were tested in the tests in the preliminary GC-MS analyses carried out with nicotine and cotinine standards. These tests were carried out to both the Scan and SIM modes of mass spectroscopy. When quinolone and 1,4-butanediol were used as internal standards, the use of high concentrations of these chemicals (at ppm level), early contamination of the inlet and column, and more importantly, the carry-over risks that may damage the calculations in repeated analyzes has been identified. In the use of the chemical 5-aminoquinoline, an overlap with the cotinine peak in the chromatogram was observed. In the use of fluorobenzene, both the reproducibility data were low and it was thought that it would be toxicologically correct to avoid using it as an internal standard, when there are possible alternatives. Considering the retention time, peak yield and reproducibility data, the best chemical in these operating conditions were obtained with the diphenylamine of the internal standard.

Preparation of standard chemical solutions

Nicotine and cotinine main standard solutions were prepared as 1 mg/mL for plasma and saliva analysis. Their

working standards prepared at 12 different concentration points which were 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10, 25, 50, 100 and 250 µg/mL. Using these working standards, nicotine and cotinine concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL were established in blank-plasma and blank-saliva samples and these samples were used in validation tests.

For urine analysis, main nicotine and cotinine standard solutions were prepared as 6 mg/mL and working solutions prepared at 12 different concentration points were 0.3; 0.6; 1.5; 3; 6; 15; 30; 60; 150; 300; 600 and 1500 µg/mL. Nicotine and cotinine concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 ng/mL were prepared in blank-urine samples and used in validation tests. Main diphenylamine standard stock solution was prepared as 6 mg/mL. Then prepared 60 µg/mL concentration of diphenylamine solution was employed as the working standard. Diphenylamine (200 ng/mL) was used as the final concentration in urine sample. All chemical standards were freshly prepared in methanol and then stored at –18°C before using in the study.

Samples collection and preparation to analysis

The urine and saliva samples were collected in previously cleaned up glass flasks. Blood samples were placed in heparinized tubes and then centrifuged at 3500 rpm for 5 minutes. Then, the collected samples were stored at –18 °C until used in the analysis. 0.5 mL samples were used for the determination of nicotine and cotinine in plasma and saliva contents, and 3 mL sample was used for analysis in urine samples. The developed and optimized solid phase extraction method is as given below: Initially, a Sep-Pak Vac (3 cc, 500 mg) solid-phase cartridge was conditioned by getting through 2 mL water and 2 mL methanol, respectively. Afterwards, the sample acidified with 0.2 mL acetic acid (1 N) solution was applied to the cartridge. Plasma proteins were precipitated by mixing with 0.2 mL (1 M) of acetic acid on the blood sample for 2 minutes and then centrifuged at 3500 rpm for 5 minutes. The resulting supernatant was fully transferred to the solid phase cartridge. Then, in the washing step the residues were removed with 3 mL water application. Finally, elution was carried out by passing 2 mL of methanol by 2 times through the cartridge. Approximately 4 mL of eluent was collected in the test tubes is evaporated by nitrogen until the nearly full dryness in a constant flow. The residue remaining at the bottom of the test tube was redissolved by 0.1 mL n-hexane by a horizontal mixture at 300 rpm for 5 minutes. Finally, the obtained extract was conveyed to a 1.5 mL sample vial and it was applied to the gas chromatography system as 1 µL volume under the splitless mode and other chromatographic conditions which given values above.

Method Validation

The developed chromatographic technique was validated in terms of selectivity, linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ) and recovery. In agreement with International Conference on Harmonization (ICH) Q2R1 guideline, the intraday and inter-day validation protocol were applied considering the reproducibility of the method in order to obtain accurate and precise measurements.[30] Quality control samples of validation tests were prepared in distilled water.

Selectivity

In order to determine of major ions of nicotine and cotinine chemical standards were applied to the instrumental analysis system under these optimized conditions given above by the Scan mode of mass spectrometry between 50–450 amu ions. In accordance with the obtained data 84*, 133 and 161 (m/z) ions and 98*, 119, 176 (m/z) ions were used in the quantitative analysis of nicotine and cotinine, respectively. Diphenylamine quantitative ions were determined as 77*, 168 and 169 (*m*/*z*) by the same method.

Linearity

After chromatographic conditions were established and optimized, calibration curves of nicotine and cotinine prepared in their matrix were plotted with 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL by the standard addition method. The linearity studies were performed with 3 replicates of analysis at each calibration point.

Accuracy and precision

Precision and accuracy of the method were carried out in 5 consecutive days. Precision, defined as relative standard deviation (RSD%), was determined by five individual replicates at three different concentrations which were low (50 ng/mL), medium (200 ng/mL) and high (1000 ng/mL) concentrations (n = 5). The accuracy, defined as the relative error (RE%) was calculated as the percentage difference between the added and found analytes quantity by 5 separate replicates both intraday and inter-day.

Sensitivity

The concentration of 5 ng/mL as the lowest calibration point, was used in the sensitivity tests of nicotine and cotinine for all biological samples. 10 quality control samples containing both analytes were prepared individually on the same day and applied by sequential analysis. All samples were prepared to the matrix based which means each sample was analyzed after its preparation in its own matrix. Nicotine and cotinine peaks were calculated with the calibration data obtained from the linearity test.

Recovery

The recovery of extraction procedures from plasma, urine and saliva was determined by comparing the pre-extraction spikes with the post-extraction of its. Five individual replicates of spiked samples at low, middle and high concentrations (50, 200, 1000 ng/mL, respectively) of nicotine and cotinine were prepared with and without ISTD. The extraction procedure was carried out as described in the sample preparation step.

Statistical analysis

Statistical analyses were performed using an IBM SPSS Statistics V.23 computer software. Analysis of nicotine and cotinine in plasma, saliva and urine samples data produced from the developed method was performed by the Spearman

Correlation Analysis test. Statistical significance level was accepted as $p<0.05$ for all analyses performed.

CONCLUSION

In this study, a GC-MS-based study was conducted on saliva, urine and plasma nicotine and cotinine levels of a sample group consisting of smokers and nonsmokers with COPD and healthy volunteers. A new solid-phase extraction method has been developed and successfully applied to real biological samples from volunteers. The linearity, accuracy, precision, sensitivity and recovery validation results obtained in accordance with the ICH Q2R1 guideline clearly demonstrated that the method is applicable to 91 volunteer biological samples. The results obtained were remarkable in terms of showing passive exposure to cigarette smoke and quantitative identification of healthy volunteers and COPD patients who declared that they did not smoke. Estimating the plasma level of both direct and indirect exposure to cigarette smoke, a well-known carcinogenic agent, on non-invasive samples is of great importance. The results obtained show that the developed method can be used safely for this purpose.

In our study, statistical tests were performed to calculate the correlation relationship between nicotine and cotinine concentrations in saliva and urine samples, which were not interventional samples, and nicotine amount in plasma and cigarette exposure. The strong correlation between the nicotine and cotinine values detected in urine and saliva samples and the values in both domestic and plasma samples has clearly shown the importance of second-hand exposure to cigarette smoke, which can still be defined as an important environmental carcinogenic agent, in terms of public health.

Ethics committee approval. The ethical permission protocol was approved by The Local Ethics Committee of Ankara University Medical School with B.30.2.ANK.020.70.01 decision number on 7 July 2010. This study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Information consent forms were obtained from the volunteers before being included in the study. Prior to inclusion in this investigation, the informed consent of research was obtained from all volunteers who contributed to the study with plasma, urine, and saliva samples. These biological samples were taken from 91 volunteers who applied to Department of Chest Diseases of Ankara University, Faculty of Medicine. Biological samples of saliva, blood, and urine were obtained from each patient simultaneously.

Acknowledgments. In this study, the laboratories of Ankara University, Institute of Forensic Sciences have used in all steps of analyses were biological sample collection, their storage and preparation for analysis, and also in the chromatographic analysis. Therefore, the authors would like to thank the Forensic Sciences Institute for their open cooperation and support from the design to the implementation of this research study. In addition, the authors thank the staff of Ankara University, Chest Diseases Department for their supplied convenience and assistance in the process from the collection of biological samples to their delivery.

REFERENCES

1. S. E. Murphy, *Chem. Res. Toxicol.*, **2017**, *30*, 410–419.
2. National Center for Chronic Disease Prevention and Health Promotion (US), Office on Smoking and Health (US), How Tobacco Smoke Causes Disease: The Biology and Behavioral Basis for Smoking-Attributable Disease: A Report of the Surgeon General. Atlanta (GA), 2010.
3. C. D. Klaassen, "Casarett & Doull's Toxicology: The Basic Science of Poisons", McGraw-Hill Education Press, 9th Edition, New York, 2018, p. 907.
4. J. Tsai, D. M. Homa, A. S. Gentzke, M. Mahoney, S. R. Sharapova, C. S. Sosnoff, K. T. Caron, L. Wang, P. C. Melstrom and K. F. Trivers, *Morb. Mortal. Wkly. Rep.*, **2018**, *67*, 1342–1346.
5. C. M. Chang, S. H. Edwards, A. Arab, A. Y. Del Valle-Pinero, L. Yang and D. K. Hatsukami, Biomarkers of Tobacco Exposure: Summary of an FDA-Sponsored Public Workshop, *Cancer Epidemiol. Biomarkers Prev.*, **2017**, *26*, 291–302.
6. P. Dhar, *J. Pharm. Biomed. Anal.*, **2004**, *35*, 155–168.
7. J. Hukkanen, P. 3rd Jacob, N. L. Benowitz, *Pharmacol. Rev.*, **2005**, *57*, 79–115.
8. T. Inukai, S. Kaji and H. Kataoka, *J. Pharm. Biomed. Anal*., **2018**, *156*, 272–277.
9. H. Kataoka, R. Inoue, K. Yagi and K. Saito, *J. Pharm. Biomed. Anal.*, **2009**, *49*, 108–114.
10. F. B. Shaik, G. Nagajothi, K. Swarnalatha, C. S. Kumar and N. Maddu, *Asian Pac. J. Cancer Prev.*, **2019,** *20*, 3617–3623.
11. A. N. Ramdzan, L. Barreiros, M. I. G. S. Almeida, S. D. Kolev and M. A. Segundo, *J. Chromatogr. A.*, **2016,** *1429*, 284–291.
12. R. Heinrich-Ramm, R. Wegner, A. H. Garde and X. Baur, *Int. J. Hyg. Environ. Health.*, **2002**, *205*, 493-499.
13. A. W. Abu-Qare and M. B. Abou-Donia, *J. Chromatogr. B Biomed. Sci. Appl.*,**2001**, *757*, 295–300.
14. H. S. Shin, J. G. Kim, Y. J. Shin and S. H. Jee, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.*, **2002**, *769*, 177–183.
15. N. Hikita, M. Haruna, M. Matsuzaki, E. Sasagawa, M. Murata, A. Yura and O. Oidovsuren, *Asian/Pacific Isl. Nurs. J.*, **2019**, *4*, 47–56.
16. WHO, Report on the global tobacco epidemic, **2019,** 17–19.
17. T. E. and K. M.-S. Nazmi Bilir and Hilal Özcebe, "Tobacco Control in Turkey Story of commitment and leadership Tobacco Control in Turkey Story of commitment and leadership", WHO, 2012, p. 1–66.
18. N. L. Benowitz, *Epidemiol. Rev.*, **1996**, *18*, 188–204.
19. J. L. Repace and A. H. Lowrey, *Risk Anal.*, **1993**, *13*, 463–475.

20. R. Huang, S. Han and X. S. Li, *Anal. Bioanal. Chem.*, **2013**, *405*, 6815–6822.
21. G. Bazylak, H. Brózik and W. Sabanty, *J. Pharm. Biomed. Anal.*, **2000**, *24*, 113–123.
22. O. A. Ghosheh, D. Browne, T. Rogers, J. de Leon, L. P. Dwoskin and P. A. Crooks, *J. Pharm. Biomed. Anal.*, **2000**, *23*, 543–549.
23. P. Zuccaro, I. Altieri, M. Rosa, A. R. Passa, S. Pichini, G. Ricciarello and R. Pacifici, *J. Chromatogr.*, **1993,***621*,257–261.
24. J.A. Thompson, M.-S. Ho and D.R. Petersen, *J. Chromatogr. B Biomed. Sci. Appl.*, **1982,** *231*, 53–63.
25. R. Bono, M. Vincenti, T. Schilirò, D. Traversi, C. Pignata, E. Scursatone, G. Dotti and G. Gilli, *J. Expo. Sci. Environ. Epidemiol.*, **2005,** *15*, 66–73.
26. C. N. Man, L.-H. Gam, S. Ismail, R. Lajis and R. Awang, *J. Chromatogr. B.* **2006,** *844*, 322–327.
27. A. E. Raducanu, C. A. G. Niculae, T. Onisei, L. G. Radu and M. A. Popescu, *Rom. Biotechnol. Lett.*, **2014,** *19*, 9485–9492.
28. P. 3rd Jacob, L. Yu, M. Duan, L. Ramos, O. Yturralde and N. L. Benowitz, *J. Chromatogr. B, Anal. Technol. Biomed. Life Sci*., **2011,** *879*, 267–276.
29. B. Karačonji, L. Skender and V. Karačić, *Acta Chim. Slov.*, **2007**, *54*, 74–78.
30. ICH Topic Q2 (R1), "Validation of Analytical Procedures: Methodology Text and Methodology", *International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use*, 2005.