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Simple, rapid and sensitive assay method for simultaneous quantification of urinary nicotine and cotinine using gas chromatography–mass spectrometry

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Abstract

Nicotine is a major addictive compound in cigarette. Its smoke is rapidly and extensively metabolized to several metabolites in human. Cotinine as a major metabolite of nicotine is commonly used as a biomarker to determine active and passive smokers. Cotinine has a longer half-life (∼20 h) compared to nicotine (∼2 h). A simple, sensitive, rapid and high throughput GC–MS method was developed for simultaneous quantification of urinary nicotine and cotinine in passive and active smokers. In the sample preparation method, the analytes and internal standard were first basified and followed by liquid–liquid extraction. Upon completion, anhydrous sodium sulphate was added to the solvent mixture to trap moistures. The clear extract obtained was directly injected into GC–MS, operating under selective ion monitoring (SIM) mode. Calibration curves in the range of 0.5–5000 ng/mL of the analytes in urine matrix were established with linear correlation coefficients (r^2) greater than 0.997. The limit of detection for both nicotine and cotinine were 0.20 ng/mL. The mean recoveries for nicotine and cotinine were 93.0 and 100.4%, respectively. The within- and between-assay accuracies were between 2.1 and 7.9% for nicotine and between 0.7 and 11.1% for cotinine. Within- and between-assay precisions of 3.3–9.5% for nicotine and 3.4–9.8% for cotinine were also achieved. The method can be used in routine assessment and monitoring of active smoking and exposure to environmental tobacco smoke. The applicability of the assay was demonstrated in a small-scale comparison study between smokers and non-smokers.

Keywords: Nicotine; Cotinine; Urine; GC–MS; Environmental tobacco smoke (ETS)

1. Introduction

Tobacco smoke is known to be the cause of several adverse health effects to both active and passive smokers [1–3]. Active smoker is referred to as the cigarette smoker whereas passive smoker is defined as the non-smokers who are exposed to environmental tobacco smoke (ETS). There are several biomarkers suggested for determination of smoking and ETS status in human, which include thiocyanate, carbon monoxide, carboxyhemoglobin, hydroxyproline, 4-aminobiphenyl, polyaromatic hydrocarbons, *N*-nitrosoproline, aromatic amine and DNA adducts. However, none of these biomarkers serve as good indicators due to lack of either specificity or sensitivity for the detection of tobacco smoke exposure [4–8]. On the other hand, nicotine, which is the main compound in the cigarette smoke is rapidly and extensively metabolized into several metabolites in human and is therefore not a suitable biomarker [9,10]. One of the major metabolites for nicotine is cotinine. Cotinine has relatively longer half-life than nicotine and it can be easily detected in urine, plasma and saliva. Urinary cotinine is a widely used biomarker due to higher concentration in urine matrix compared to other matrixes, thus could be detected accurately in urine [11,12].

There are numerous types of assays used to quantify urinary nicotine and cotinine levels, namely, the radioimmunoassays [13,14] and enzyme-linked immunosorbent assays [12,15]. A wide range of equipments have also been used to determine these urinary nicotine and cotinine which include colorimetry, gas chromatography and high-performance liquid chromatography [16–19]. However, none of these assays offer either high specificity or sensitivity for detection of the compounds. In order to detect urinary cotinine in passive smoker accurately and

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reliably, one assay should achieve a limit of quantitation (LOQ) of at least 1 ng/mL, which is free from caffeine interference [20]. As such, a couple of assays, applying mass spectrometer coupled with gas chromatograph or liquid chromatograph were developed. The limit of detection (LOD) within the range of 0.2–0.6 ng/mL and LOQ within the range of 1–20 ng/mL for nicotine and cotinine were reported by the authors [20–27].

General procedure for extraction of analytes normally involves either liquid–liquid extraction (LLE) [18–21,23,24] or solid-phase extraction (SPE) [25–31]. The extract obtained was evaporated to dryness or to salting the analyte prior to chromatography analysis. In proposed developed method, extraction procedure was improved by using LLE, which reduced the cost of analysis as compared to that of the SPE method. The extraction method was also simplified and therefore could be performed in a single tube. Furthermore, only 0.5 mL of organic solvents was used in the extraction procedure. The extract was directly injected into GC–MS without undergoing evaporation or salting steps. In addition, a relatively shorter analysis time was applied. Through validation processes, the method was evident to be simple, sensitive, rapid and high throughput for simultaneous quantification of urinary nicotine and cotinine. The applicability of the assay was demonstrated in a study involving a group of male students (242 respondents) from the Universiti Sains Malaysia (USM), Penang consisted of 44 smokers and 198 non-smokers. The subjects were interviewed using questionnaire prior to the collection of their urine sample.

2. Experimental

2.1. Materials

All chemicals were of analytical grade and obtained from several sources: S(−)-nicotine and diphenylamine (Sigma), (−)-cotinine (Fluka), methanol and chloroform (J.T. Baker), anhydrous sodium sulphate (Merck) and sodium hydroxide (Surechem). The purified water was of MilliQ (Millipore) quality.

2.2. Blank, standards and quality controls

Urine samples from non-smoker volunteers who claimed to be free from cigarette smokes for the past 5 days were collected, extracted and analyzed. Urine samples with non-detectable nicotine and cotinine were pooled and used in the preparation of calibration and quality control samples (QCs). A stock solution containing 1 mg/mL of nicotine and cotinine in methanol was prepared. Three working solutions (1, 10 and 100 μg/mL) were prepared from the stock solution. A set of eight calibrators made up of 0.5, 10, 50, 100, 500, 1000, 2500 and 5000 ng/mL nicotine and cotinine in urine was prepared daily from working solutions. Three QCs (0.5, 2500 and 5000 ng/mL) of nicotine and cotinine in urine were also prepared from a separate stock solution (1 mg/mL). The internal standard (I.S.), diphenylamine working solution (250 ng/mL) was prepared in methanol. All solutions, blank and QCs were stored at −20 °C prior to analysis.

2.3. Sample preparation

One milliliter each of purified water, blank urine, samples, QCs and calibrators was pipetted into separate tubes. Internal standard (0.175 mL), 0.1 M sodium hydroxide (0.050 mL) and chloroform (0.325 mL) were then added. Up to this point, each of the tube will contain a total volume of 1.550 mL mixture comprising 1.050 mL aqueous solution and 0.500 mL organic solvents (methanol:chloroform in the ratio of 1:1.9). The mixture was vortexed at 1500 rpm for 1 min and centrifuged at 2500 rpm for 4 min. The aqueous layer was discarded. A small amount of anhydrous sodium sulphate (∼0.1 g) was added and mixed briefly. After leaving at room temperature for 1 min, the clear organic extract was then transferred into an auto sampler vial.

2.4. Gas chromatography–mass spectrometry conditions

GC–MS analyses were performed on HP6890 GC coupled with HP5973 mass spectrometer detector. The column was fused-silica capillary, HP-5MS column (30 m × 0.25 mm i.d.; 0.25 μm film thickness) and helium was used as a carrier gas at a constant pressure of 14.5 psi. One microliter injection volume using splitless mode was performed on injector temperature at 250 °C. The oven temperature was programmed from 70 to 230 °C (1 min hold) at a rate of 25 °C/min. Post run was set at 310 °C for 6 min. The total run time was 7.4 min. The interface temperature was set at 280 °C. Selective ion monitoring (SIM) mode was used in the analysis. Four ions in a group at *m/z* 162, 161, 84, 133, and *m/z* 173, 118, 119, 98 were used to monitor nicotine and cotinine, respectively, and for I.S., the ions were at *m/z* 169, 141, 77. Quantification was based on the peak area integration at *m/z* 162 (nicotine), 176 (cotinine) and 169 (I.S.). The other ions served as qualifying ions.

2.5. Linearity and sensitivity

The linearity of the assay was calculated based on the regression line by the method of least squares and expressed as the correlation coefficient ($r^2 > 0.995$). A $1/x$ weighting factor was applied and linearity of each analyte was determined using the eight calibrators. The linearity of the curves was accepted when each of the calibrators achieved concentration not exceeding 20% of the nominal (actual) concentration. Calibration curves were constructed on peak area ratio of analyte/internal standard versus concentrations using linear regression. The data were analyzed using Microsoft Excel 2002 (10.6501.6626) SP3 software.

LOD is the lowest concentration of an analyte determined with signal to noise ratio of at least 3:1 by peak height. LOQ is the lowest concentration of an analyte in a calibration curve and it may use the criteria of LOD (ratio 3:1). The accuracy and precision of the LOQ (at least six replicates) must be <20% of the nominal concentration.

2.6. Precision and accuracy

Within- and between-assay precision and accuracy were calculated by using low (0.5 ng/mL), medium (2500 ng/mL) and

high (5000 ng/mL) QC samples. Within-assay precision and accuracy were determined by comparing data from within one run ($n = 6$ for each concentration), while between-assay precision and accuracy were determined by comparing data between three runs. Precision was expressed as percent coefficient of variation and accuracy as the percent difference from the nominal values.

2.7. Recovery

The recovery was determined by comparing the mean peak area ratio of urine extracts containing nicotine and cotinine at 0.5, 2500 and 5000 ng/mL ($n = 6$ for each concentration) with the non-extracted standards in methanol.

2.8. Specificity

Potential interference was evaluated against the commonly used drugs, which was randomly selected from prescribed or non-prescribed drugs that are easily obtainable over the counter. The study was carried out to determine the possible interference of these drugs with the detection of nicotine and cotinine. Ten drugs were spiked into blank and sample urine to achieve concentration of 0.2 μg/mL (amitryptyline, bromhexine, chlorpheniramine, trifluperazine), 0.5 μg/mL (dothiepin) and 10 μg/mL (clomipramine, propranolol, sulpiride, verapamil, isosorbide dinitrate). These concentrations were much higher than their therapeutic levels in plasma [32].

The assay may be considered specific or free from possible interference if:

- In blank urine, any of the spiked drugs were eluted at retention times outside the region of ±0.2 min retention times of both analytes or any of the spiked drugs were detected within that region, the peak height were less than 2% of that of 0.5 ng/mL calibrator.
- In sample urine, the addition of drugs did not change the analytes retention times >2% and their peak height ratios (qualifying ions to quantitative ion) >20%.

2.9. Application

Urine samples were collected from 242 male student volunteers from USM. The history of smoking or exposure status was recorded in questionnaire. Urine samples were collected in polypropylene containers and stored at −20 °C prior to analysis. The samples were extracted and analyzed according to the recently developed assay.

3. Result and discussion

The extraction of nicotine and cotinine using LLE was reported previously by several authors [18–21,23,24]. In addition, the use of SPE in sample preparation was claimed to simplify, and reduce extraction time and solvent consumption as compared to LLE. These assays used commercialized cartridges like Extrelut [25], Oasis HLB [27], Drug Test-1 [28], C18 Isolute [29] and Amberlite XAD-2 [31]. Both assays (LLE and SPE) used large volume (2–15 mL) of organic solvents which were then evaporated in order to recover the analytes. However, nicotine is a very volatile compound compared to cotinine; nevertheless, both can be easily lost during evaporation. Thus, solvent selection and evaporation are very critical in nicotine–cotinine sample preparation. Evaporation of the solvent should be done with extra caution to prevent over drying, which may result in the loss of the analytes. In order to reduce the volatility of nicotine during evaporation, acids such as hydrochloric acid, acetic acid, phosphoric acid and sulphuric acid were used to form nicotine salts [21,25,34–36]. In the presently developed method using LLE assay, the extraction was performed in a single tube with only a small volume of organic solvents, i.e. 0.175 mL methanol (from I.S.) and 0.325 mL chloroform. The evaporation of the solvent and salting was not necessary because extremely small volume of solvent was used and the mixture of the solvents was at the correct composition for GC–MS analysis. As a result, recovery of both nicotine (93.0%) and cotinine (100.4%) (Table 1) was dramatically improved compared to other published assays [23,25,27–29]. Using such extraction method, we have simpli-

Table 1
The assay precision, accuracy and recovery of nicotine and cotinine

Analyte	Conc. (ng/mL)	Within-assay[a]			Between-assay[b]			Recovery[a] (%)
		Observed conc. (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)	Observed conc. (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)	
Nicotine	0.5	0.54 ± 0.05	9.5	7.9	0.52 ± 0.04	7.8	6.5	100.9
	2500	2553.68 ± 83.39	3.3	2.1	2538.38 ± 171.46	6.8	6.5	95.8
	5000	4745.10 ± 364.14	7.7	5.1	5103.90 ± 257.16	5.2	5.5	82.4
Average			6.8	5.0		6.6	5.6	93.0
Cotinine	0.5	0.50 ± 0.05	9.8	0.7	0.52 ± 0.04	8.5	4.4	104.4
	2500	2763.76 ± 92.63	3.4	10.6	2777.36 ± 111.06	4.0	11.1	101.8
	5000	4656.96 ± 392.27	8.4	6.9	4906.71 ± 288.10	5.9	5.4	95.1
Average			7.2	6.1		6.1	7.0	100.4

[a] $n = 6$ for each concentrations.
[b] $n = 18$ for each concentrations.

fied and reduced the cost and time for the sample preparation. Therefore, quantification of nicotine and cotinine can be performed in a non-laborious and cost-effective way as compared to other assays [21–31,35,36].

The high sensitivity achieved by this assay is critically important, as our primary interest is not only to monitor active and passive smokers but also the subjects who may not expose to ETS. The total ion chromatogram of the analytes appeared clean

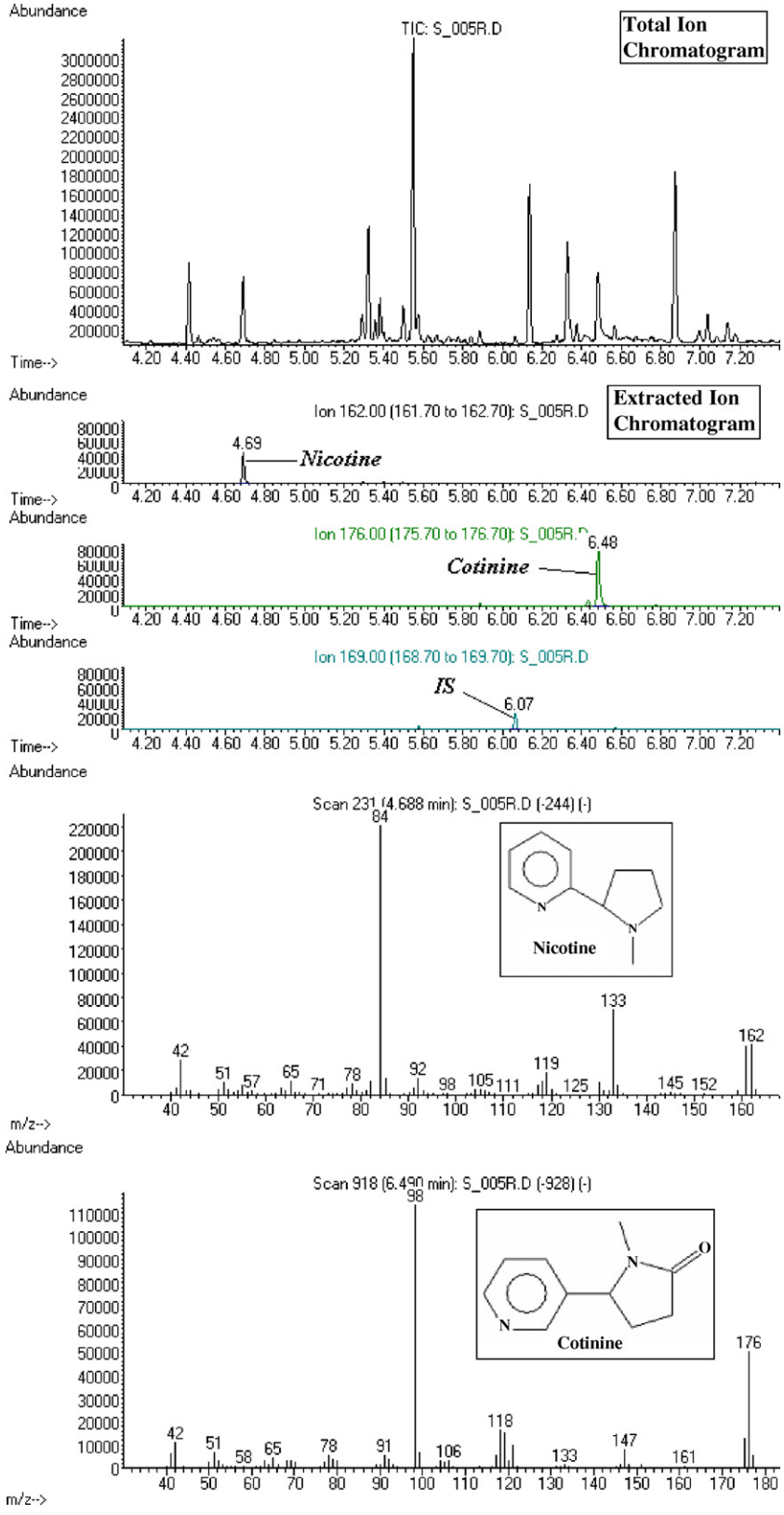

Fig. 1. Chromatograms and mass spectra of nicotine and cotinine of a smoker.

from the interference that derived from the urine matrix. The examples of total ion chromatogram (TIC), extracted ion chromatogram (EIC) and mass spectra of nicotine and cotinine of a smoker are shown in Fig. 1. The clean ion chromatograms and high recovery have greatly contributed to the low detection limits that were attainable by our assay. The LOD and LOQ of the assay for both nicotine and cotinine were achieved at 0.2 and 0.5 ng/ml, respectively.

In the MS analysis, quantification was based on the intensity of the molecular ions of the analytes. The molecular ions chosen were the ions with the highest *m/z* value for each analytes, having the minimum background interferences or chemical noises compared with their corresponding lower mass fragments. Therefore, producing better peak shapes for quantification purposes. With the same analogy, the use of such molecular ions as quantification markers have substantially contributed to the sensitivity of the assay, whereby it improves the LOQ of the assay as compared to other methods [12,20,21,23,24,33].

The linearity of the assay with an average of correlation coefficient $r^2 > 0.998$ was achieved for the entire 3 days of validation period over a dynamic range of 0.5–5000 ng/mL for each of the analytes. The wide linear range and the high sensitivity of this assay have enabled them to be used reliably on urine samples from smokers, non-smokers and people exposed to ETS. Within-assay precision and accuracy were less than 10% for nicotine and 11% for cotinine. Between-assay precision and accuracy were less than 9 and 12% for nicotine and cotinine, respectively (Table 1). A good separation chromatogram for the analytes was achieved at relatively shorter analytical time, for nicotine and cotinine at 4.69 and 6.48 min, respectively. Total GC–MS run time was also shortened to 7.4 min as compared to others (8–10 min) [20,23,33]. Furthermore, the assay had offered great specificity as no interference was observed from all the 10 drugs tested.

Interference from caffeine is a common problem accounted in most of the cotinine analysis methods based on chromatography separation [25], in which caffeine is normally co-elute with cotinine. This interference becomes immense when very low amount of cotinine is measured. In the present GC separation method, the temperature ramping used allows caffeine to be eluted at 7.3 min which was much later than the elution of cotinine, and thus does not interfere with its detection.

4. Application

In this study, two groups of self reported smokers were identified based on their cotinine levels. The first group (93.2%) had cotinine level ranging from 100 to 3550 ng/mL whilst nicotine ranging from 1 to 1695 ng/mL. The second group (6.8%) had lower levels of nicotine and cotinine, less than 0.5 and 10–14 ng/mL, respectively. Lower cotinine was recorded in the second group as they claimed, not smoking for at least 6 days before the urine were collected. Cotinine half-life is reported to be ∼20 h and 100 ng/mL urinary cotinine is known as a cut-off for active smokers [25]. Data of the current analysis shows that nicotine is not an appropriate marker to determine smoker status as it has a much shorter half-life (∼2 h) which results in a wide range of nicotine levels that do not correlate with the smoking status of the smokers.

Hundred percent (or 198 subjects) of self reported non-smokers had cotinine level ranging from 0 to less than 23 ng/mL. This range was well below smoker cut-off level. 86.4% of these non-smokers had cotinine less than 5 ng/mL, a value that indicates non-exposure to ETS [36,37]. The data indicated that only 13.6% were exposed to ETS. All the non-smokers had nicotine levels <5 ng/mL.

5. Conclusion

We have developed a high throughput method for simultaneous quantification of urinary nicotine and cotinine. The method is rapid, sensitive, accurate and simple. The extraction of the analytes was carried out in a single tube with the use of very little solvents. The procedure was also simplified by omitting the evaporation and salting of the analytes, which are the critical steps in other analysis methods. The GC–MS analysis was carried out in 7.4 min with no interference from caffeine. The results indicated that cotinine level serve as a useful biomarker for tobacco exposure. Hence, it is applicable for routine assessment and monitoring of active smoking and exposure to environmental tobacco smoke. The applicability of the assay was successfully demonstrated in a small-scale comparison study between smokers and non-smokers.

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References

[1] R. Peto, A.D. Lopez, J. Boreham, M. Thun, C. Heath Jr., Indirect Estimates from National Vital Statistics, Oxford University Press, 1994.
[2] J.R. DiFranza, R.A. Lew, Pediatrics 97 (1996) 560.
[3] International Agency for Research on Cancer (IARC). IARC monographs on the evaluation of carcinogenic risks to humans and their supplements: tobacco smoke and involuntary smoking, vol. 83, 2002.
[4] N.J. Haley, C.M. Axelrad, K.A. Tilton, Am. J. Public Health 73 (1983) 1204.
[5] J.C. Hauth, J. Hauth, R.B. Drawbaugh, Obstetr. Gynecol. 63 (1984) 519.
[6] M. Jarvis, H. Tunstall-Pedoe, C. Feyerabend, C. Vesey, Y. Salloojee, Am. J. Public Health 77 (1987) 1435.
[7] F.G. Crawford, J. Mayer, R.M. Santella, T.B. Cooper, R. Ottman, W.Y. Tsai, G. Simon-Cereijido, M. Wang, D. Tang, F. Perera, J. Natl. Cancer Inst. 86 (1994) 1398.
[8] E.M. Sharon, A.L. Carrie, J. Joni, L. Chap, S.P. Susan, S.H. Stephen, G.C. Steven, L. London, K.H. Dorothy, Cancer Epidemiol. Biomarkers Prevent. 13 (2004) 1617.
[9] A. Pilotti, Acta Physiol. Scand. Suppl. 479 (1980) 13.
[10] D.E. Robinson, N.J. Balter, S.L. Schwartz, J. Pharmacokinet. Biopharm. 20 (1992) 591.
[11] R.L. Repace, A.H. Lowney, Risk Anal. 13 (4) (1993) 463.
[12] H.W. Kuo, J.S. Yang, M.C. Chiu, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 768 (2) (2002) 297.

[13] Z. Piergiorgio, P. Simona, A. Ilaria, R. Mirella, P. Manuela, P. Roberta, Clin. Chem. 43 (1997) 180.
[14] J.J. Langone, H. Van Vunakis, Methods Enzymol. 84 (1982) 628.
[15] R.J. Bjercke, G. Cook, N. Rychlik, H.B. Gjika, H. Van Vunakis, J.J. Langone, J. Immunol. Methods 90 (1986) 203.
[16] E.V. Puhakainen, R.D. Barlow, J.T. Salonen, Clin. Chim. Acta 170 (2–3) (1987) 255.
[17] J. Moore, M. Greenwood, N. Sinclair, J. Pharm. Biomed. Anal. 8 (1990) 1051.
[18] F. Ceppa, Y. El Jahiri, H. Mayaudon, O. Dupuy, P. Burnat, J. Chromatogr. B Biomed. Sci. Appl. 746 (2000) 115.
[19] M. Hariharan, T. VanNoord, Clin. Chem. 37 (7) (1991) 1276.
[20] J.S. Torano, H.J. van Kan, Analyst 128 (7) (2003) 838.
[21] H.S. Shin, J.G. Kim, Y.J. Shin, S.H. Jee, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 769 (1) (2002) 177.
[22] J.T. Bernert, T.L. Harmon, C.S. Sosnoff, J.E. McGuffey, J. Anal. Toxicol. 29 (8) (2005) 814.
[23] A.J. Ji, G.M. Lawson, R. Anderson, L.C. Dale, I.T. Croghan, R.D. Hurt, Clin. Chem. 45 (1999) 85.
[24] H. James, Y. Tizabi, R. Taylor, J. Chromatogr. B Biomed. Sci. Appl. 708 (1–2) (1998) 87.
[25] T. Tapani, J. Tom, R. Kari, Clin. Chem. 45 (1999) 2164.
[26] D.L. Heavner, J.D. Richardson, W.T. Morgan, M.W. Ogden, Biomed. Chromatogr. 19 (4) (2005) 312.
[27] X. Xu, M.M. Iba, C.P. Weisel, Clin. Chem. 50 (12) (2004) 2323.
[28] P.B. Doctor, V.N. Gokani, P.K. Kulkarni, J.R. Parikh, H.N. Saiyed, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 802 (2) (2004) 323.
[29] E.E. Baidoo, M.R. Clench, R.F. Smith, L.W. Tetler, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 796 (2) (2003) 303.
[30] J. Yang, Y. Hu, J.B. Cai, X.L. Zhu, Q.D. Su, Anal. Bioanal. Chem. 384 (3) (2006) 761.
[31] K. Tyrpien, T. Wielkoszynski, B. Janoszka, C. Dobosz, D. Bodzek, Z. Steplewski, J. Chromatogr. A 870 (1–2) (2000) 29.
[32] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop (Eds.), Clarke, Isolation and Identification of Drugs, second ed., The Pharmaceutical Press, London, 1986.
[33] M. Culea, O. Cozar, S. Nicoară, R. Podea, Indoor Built Environ. 14 (2005) 293.
[34] H. Van Vunakis, H.B. Gjika, J.J. Langone, IARC Sci. Publ. 81 (1987) 317, Abstract-MEDLINE.
[35] O. Christiane, C.D. Jean, B. Monique, Clin. Chem. 45 (1999) 505.
[36] H. Dietrich, L.W. Ernst, Tobacco smoke biomarkers of the uptake of ETS by non-smokers, ILO, Encyclopedia of Occupational Health and Safety, fourth ed., 2002.
[37] V. Haufroid, D. Lison, Int. Arch. Occup. Health 71 (1998) 162.