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Comparison between the Nicotine Content in Smokers and Non-Smokers' Blood Samples Collected from some Volunteers in El-Beida City, Libya Using RP-HPLC

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ABSTRACT

Cigarette smoking is the main cause of death and disease in our society. It, clearly has a dangerous impact on the health of smokers and other people around them, alongside economic issues for the smokers. Nicotine is merely one among the several thousands of compounds identified in tobacco. The aim of this study was to determine of nicotine in blood samples in smokers and nonsmokers by developement of a rapid, simple, reliable, and one-step extraction method, in order to isolate and determine nicotine in human plasma samples using Reversed Phase - High Performance Liquid Chromatography (RP-HPLC), constituting liquid–liquid extraction with binary solvents to get better detection limit, linearity over high range, recovery, and no interference peaks. In the present study, twenty blood samples are collected from smokers and nonsmokers in El-Beida City, Libya. Samples are taken from an volunteer at the same time after each volunteer fills during a questionnaire. The method of analysis is validated over a wide linear range of 1.62–162.12 µg/mL with correlation coefficients being consistently greater than 0.9999. The results of nicotine concentrations in male smokers' plasma are within the range of 3.292 - 66.398 µg/mL with an average of 11.950 µg/mL. Whereas its concentrations in non-smokers'plasma are in the range of 3.3004 - 9.001 µg/mL with an average of 4.624 µg/mL. The average of the concentrations of nicotine in smokers'plasma is greater than non-smokers plasma. The criteria considered for validation are: limit of detection, limit of quantitation, linearity, accuracy, precision and confidence limit. Statistical analysis show that the nicotine levels were significant difference within the smoker samples in contrast with the nonsmoker samples using RP-HPLC method.

Introduction

Cigarette smoke may be a complex mixture of gaseous and particulate matter. More than 4,700 chemical compounds, including 43 cancer-causing substances, have been isolated in cigarette smoke (Bartecchi et al., 1995). Individually and in combination, the particulate and gaseous compounds found in cigarette smoke inhibit the conditions required for expeditious wound repair (Silverstein, 1992). As the recognition of such problems has become more widespread, the movement to ban smoking has gained momentum (Tollinson, 1988; Watson and Witten, 2001). Studies that have specifically looked at adolescent smoking in relation to various individual or family characteristics have found that Whites are more likely to smoke compared to other racial/ethnic groups (Centers for Disease Control and Prevention, 2003; Orlando et al. 2004; Wills and Cleary, 1997). In addition, nonsmokers are more likely than consistent smokers to return from intact nuclear families or from families with more highly educated parents (Orlando et al. 2004). Smoking has been explosively intertwined as a threat factor for chronic obstructive pulmonary disease, cancer and atherosclerosis, etc. (Gupta et al., 1997 ; Padmavati, 2002). The World Health Organization estimates that deaths resulting from cogarette smoking in India might exceed 1.5 million annually by 2020 (Rani et al., 2003 ; Pasupathi et al., 2009). The highly toxic chemical in tobacco alkaloids is nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine present within the leaves of common tabacco (Wu et al., 1998 ; Rodricks , 1992). Nicotine is only one of many thousands of compounds identified in tobacco that make up tobacco contributing to its flavor, aroma, and physiological effects. Nicotine is a tracer for environmental tobacco smoke (ETS) due to the fact that it specifies the tobacco (Jones, 1994). In addition, it is a chemical that is commonly used as a natural insecticide, asa well as being a highly addictive drug (Hamm, 1982). Cotinine is the major primary metabolite of nicotine (Xu et al., 1996 ; Zevin et al., 1997), and it accumulates in the body with regular smoking. Nicotine and cotinine

appear to be metabolized by the same liver enzyme (Zevin et al., 1997). Nicotine has a fairly short half-life, approximately 2 h, and cotinine has a half-life of roughly 20 h (Benowitz, 1983). The true smoking status is based on cotinine and nicotine levels in the body fluids (Shin et al., 2002) that are dependent on sex, age, diet, racial and ethnic differences, as well as many other factors (Tyrpien et al., 2000). Assessments of nicotine and cotinine in biological fluids like blood, urine, and other biological markers have become an important component of direct or passive exposure to tobacco smoke (Doctor et al., 2004). Nicotine and cotinine in biological samples are often detected using different instrumental techniques. For example, high-performance liquid chromatography (HPLC) using UV detector (Oddoze et al., 1998) or mass spectrometry detector (MS) (Davoli et al., 1998). Other studies have determined nicotine and/or cotinine levels in blood and/or urine samples in smokers and nonsmokers by HPLC and/or gas chromatography (GC) - MS (Torano and Van Kan, 2003 ; Heavner et al., 2005 ; Massadeh et al., 2009; Elmanfe et al., 2019; Elmanfe et al., 2020) or by a simple method for determination of nicotine in smokers and nonsmokers'plasma by UV-Visible spectrophotometer (Elmanfe and Abdulla, 2014). In our study, a rapid, simple, reliable, and one-step extraction method is developed, based on other methods recommended by other researchers with some modifications, in order to isolate and determine nicotine and cotinine in human plasma in smokers, constituting liquid–liquid extraction with binary solvents (Davoli et al., 1998 ; Massadeh et al., 2009; Elmanfe and Abdulla, 2014) to get better detection limit, linearity over high range, recovery, and no interference peaks. The extraction method used is more rapid and simple compared with other extraction methods (Ceppa et al., 2000). Another advantage of this method is that it utilizes one extraction step with 5-10 mL of a solvent mixture. The analyses of the method were all developed and validated by HPLC. This study was aimed to estimate the levels of nicotine and detection of cotinine in smokers and nonsmokers'plasma samples using RP-HPLC.

Experimental

1. Chemicals and reagents:

The chemicals, analytical standards, reagents, and solvents used throughout this study were analytical grade and highly pure. Nicotine was purchased from (Fluka) with purity of Assay ≥ 99% (for research and development). Methanol (HPLC/SPECTRO) was purchased from (Sigma-Aldrich) with purity 99.9 % Assay (GC). Dichlormethane (Riedel-Dehaen AG Seelze Hannover) with purity 99.5 %; potassium dihydrogen phosphate (Riedel-Dehaen AG Seelze Hannover), with purity 99%); ortho-phosphoric acid (Merck, 89% assay); diethylether (Sigma-Aldrich, Chromasolv, for HPLC) with purity 99.9 % inhibitor-free; sodium hydroxide from (Riedel-Dehaen AG Seelze Hannover); hydrochloric acid (Merck).

2. preparation of standard solutions:

a)- Stock nicotine solution: (Fluka): 50 mg in 100 mL (0.5 mg/mL) solution was prepared. Standard nicotine solutions were prepared by appropriate dilution of the stock. (1.62, 16.21, 48.64, 81.06 and 162.12 µg/mL). *b)- Potassium dihydrogen phosphate (KH_2PO_4) :* 0.2973 g of KH_2PO_4 was dissolved in 1 L or 0.5946 g of KH_2PO_4 in 2 L. This solution was used as mobile phase for HPLC, The pH of the mobile phase was adjusted by drop wise addition of ortho-phosphoric acid (pH ≈ 3.2). c)- *2.5 M sodium hydroxide (NaOH):* 10 g of sodium hydroxide was dissolved in distilled water to make 100 mL or (1g in 10 mL). *d)- 0.25 M hydrochloric acid*: 2 mL of concentrated hydrochloric acid were diluted with distilled water to 100 mL.

3. instrumentation:

The HPLC system (Perkin Elmer Series 200 Pump) Autosampler, Series 2000 UV/Vis Detector, The Series 200 Autosampler, Series 200 Analytical Pump, Series 200 Column Oven, and 10 µL loop injector. The stationary phase represents the analytical column was a Brownlee Bio C18 column of 250x4.6 mm and 5 µm particle size.

HPLC operating conditions :

Mobile Phase:
A: 82% phosphate buffer (KH_2PO_4) ; pH ≈ 3.2.
B : 18% methanol.
Flow rate: 1 mL/min. *Injection*: 10 µL.
Tr: 4 min for nicotine.

4. Standard Solutions (HPLC Calibration) :

Calibration standards in the range 1.62-162.12 µg/mL were prepared by serial dilution from the 500 µg/mL mixed standard as showed in (figure 1). Standard solutions were stored at -4 °C.

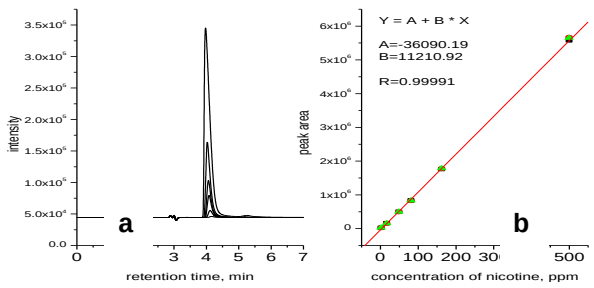

Figure (1) : Chromatograms of different concentrations of nicotine at 258 nm (a);

Calibration curve for nicotine, expressed on a linear scale (b).

5. Samples collection :

Twenty samples were collected from Medical Laboratory of Clinic of Omar Al-Mukhtar University, El-Beida- Libya. Eight of those samples were collected from male smokers, ten from male non-smokers and two from female non-smokers. Blood samples were taken from the same person at the same time after each volunteer filled out a questionnaire. The questionnaire included, the age, the time of the sample collection, the number of cigarettes smoked and the kind of cigarettes. The (table 1) show the sample collection from male smokers'plasma ; and the (table 2) show the sample collection from male and female nonsmokers'plasma.

Table (1): Sample collection from mal smokers'plasma

S. No.	Age/year	Smoking period / year	cigarettes brands	Amount smoked/daily	Time/min
1	49	26	unknown	Two Pocket	15 min
2	60	40	Ors	One pocket	1 h and 20 min
3	37	25	---------	Two pocket	10 min
4	34	14	----------	half Pocket	Unkown
5	18	4	Red Karelia	Two Pocket	----------
6	27	15	White Karelia	Two pocket	15 min
7	25	4	Blue Karelia	half Pocket	10 min
8	26	14	Malbory	Pocket and half	2 min

S.No = Sample Number; Time/min = After Smoking

Table (2): Sample collection from male and female nonsmokers'plasma.

Sample Number	volunteer Age / year	Volunteer Gender
9	46	Male non smoker
10	65	Male non smoker
11	42	Male non smoker
12	29	Male non smoker
13	26	Male non smoker
14	50	Male non smoker
15	55	Male non smoker
16	67	Mal non smoker
17	27	Male non smoker
18	21	Male non smoker
19	22	Female non smoker
20	22	Female non smoker

6. Blood samples:

The blood samples (4 mL of each sample) were collected in EDTA-tubes (Vacuette EDTA K2/gel tube) and centrifuged immediately at 2800 rpm for 5 min. The plasma supernatant was then collected in eppendorf tubes in the laboratory at Medical Laboratory of Clinic of Omar Al-Mukhtar University, El-Beida- Libya and frozen at –70°C until analysis.

7. Extraction of Nicotine:

The extraction procedures were carried out with a slight modification based on other researches. (Massadeh et al., 2009; Elmanfe and Abdulla, 2014; Elmanfe et al., 2019). A 0.5 mL aliquot of plasma was placed into a screwcapped glass tube 15 × 100-mm. Each sample was alkalinized with 200 µL of 2.5 M NaOH, then vortex mixed at 2800 rpm for 2 min. A 5-10 mL aliquot of dichloromethane–diethylether (1:1 v/v) was used for one-step single extraction, then vortex mixed at 2800 rpm for 2 min. The organic layer, after being centrifuged at 3000 rpm for 2-4 min, was transferred to a replacement glass tube containing 40 µL of 0.25M HCl. The organic phase was then evaporated under a stream of nitrogen at 35ºC until dryness and reconstituted to 250 - 1000 µL with mobile phase consisting of a mixture of 0.2973 g of KH_2PO_4, 820 mL of water (HPLC-grade), and 180 mL of methanol (HPLC-grade). A 10-50 µL aliquot was injected utomatically into the HPLC.

Results and discussion

Results in smoker's plasma for nicotine in plasma were in the range of 3.292–66.398 µg/mL with an average of 11.950 µg/mL. these results are shown within the (table 3). For non-smokers, nicotine in plasma was in the range of 3.3004–9.001 µg/mL with an average of 4.624 µg/mL, these results are shown in the (table 4).

Table (3): The amount of nicotine in male smokers'plasma by HPLC.

Sample No.	Age / year	collection Time / min	Concentraion of nicotine /ppm (µg/mL) in 0.5 ml of Plasma
1	49	15	66.3983
2	60	80	3.2915
3	37	10	3.3913
4	34	Unkown	4.2573
5	18	----------	4.2782
6	27	15	4.3903
7	25	10	4.2624
8	26	2	5.3341

Table (4): The amount of nicotine in male and female non- smokers'plasma by HPLC.

Sample No.	Age / year	Gender	Concentraion of nicotine /ppm (µg/ml) in 0.5 ml of Plasma
9	46	male	9.0035
10*	65	male	10.5913*
11	42	male	3.3004
12	29	male	4.2111
13	26	male	4.2676
14	50	male	4.2601
15	55	male	4.2469
16	67	male	4.1678
17	27	male	4.1741
18	21	male	4.3176
19	22	female	4.2771
20	22	female	4.6373

The results obtained from HPLC in the (table 3) and (table 4) show that there was no significant difference in nicotine concentrations in plasma between smokers and non-smokers at 95% confidence level, but on average, the concentration of nicotine in smokers'plasma is greater than non-smokers plasma.

***** We have added 100 µL of the standard solution of nicotine with the concentration of 162.12 µg/mL to the plasma sample number 10*. to make sure the efficiency and validity of the extraction process (as showed in (figure 3) and (figure 4). The (figure 3) shows the chromatogram of the standard solution of nicotine has a concentration of (162.12 µg/mL) alone (without plasma sample). The (figure 4) show the chromatogram of nicotine its concentration is 162.12 µg/mL added to the non-smokers' plasma sample number **10**.

The HPLC Chromatogram for nicotine and cotinine* extracted from male smokers' plasma sample (number 5 in (table 1) and (table 3) is shown in (figure 5).

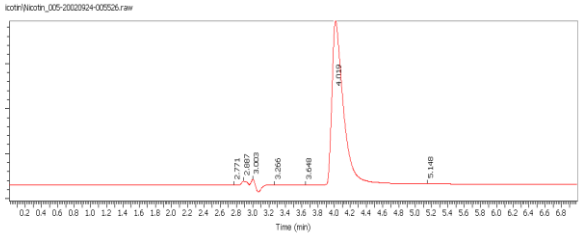

Figure (3): Chromatogram of standard solution of nicotine alone its concentration is (162.12 µg/mL).

$$LOD = \frac{3 s_{y/x}}{b}$$

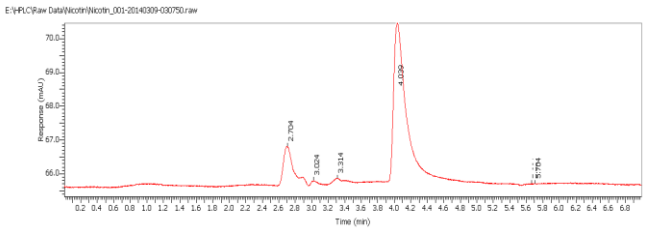

Figure (4): Chromatogram of nicotine (162.12 µg/mL) + non-smokers' plasma sample (No. **10**).

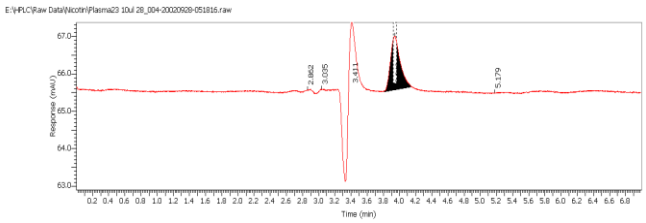

Figure (5): Chromatogram of nicotine and cotinine* in male smokers' plasma sample (number 5 in the table (1) and table (3)).

***We have supposed that the first peak appears in (figure 5) indicate the presence of cotinine in the smoker's plasma sample according the other methods and studies using HPLC (Massaden et al.. 2009 ; Torano and Van Kan. 2003 ; Nakajima et al.. 2000). In our study. we had problem to get the standard solution of cotinine and for this reason we didn't sur the results in smokers'plasma for cotinine.

Statistic study :

1. Linearity : Examination of calibration curves by computing a linear least-squares regression analysis on the plot of the peak area ratios and absorbances of nicotine to the external standard versus concentrations demonstrated a linear relation over the range 1.62 - 162.12 µg/mL in the case RP-HPLC (using six concentration levels) with correlation coefficients (R2) being consistently greater than 0.9999.

2. Limit of detection (LOD) : Is defined as the concentration of analyte required to give a signal equal to three times the standard deviation of the blank. The LOD was calculated using the following equation (Alkhamaisah et al., 2019; Pereira et al., 2021):

where s is the average of the standard deviation SDyx of the peak ratio (peak area of analyte/ peak area of external standard), and b is the average of the slope of a calibration curve. In the presented study, the limit of detection (LOD) value for nicotine in plasma samples using HPLC was 2.47 µg/mL.

3. Limit of quantitation (LOQ) : is defined as the concentration of analyte required to

give a signal equal to ten times the standard deviation of the blank. The LOD was calculated using the following equation (Alkhamaisah et al., 2019; Pereira et al., 2021):

$$LOQ = \frac{10 \cdot s_{y/x}}{b}$$

The limit of quantitation (LOQ) value for nicotine in plasma sample in HPLC was determined to be 8.24 µg/mL.

4. Accuracy and precision : Accuracy is expressed as percent relative error (% R.E.). Precision is expressed as percent relative standard deviation (% RSD). In our study the accuracy (% R.E.) was 1.065 % and the precision (% RSD) was 0.818 %.

5. Confidence Limit (or Interval) for the Mean: Is the limit (above and below) around x that µ must lie, with a given degree of certainly (or probability or confidence level).

$$x_t = \overline{x} \pm \frac{ts}{\sqrt{n}}$$

In our study, the Confidence Limit for the Mean for nicotine in smokers'plasma samples using RP-HPLC was xt= 11.950 ± 0.5721; whereas its value for nicotine in non-smokers'plasma samples was xt= 4.624 ± 0.2929.

Conclusions

The concentrations of nicotine in plasma were less than expected among a lot of people, but the average concentrations of nicotine in the male smokers' plasma samples were higher than the concentrations in male and female non-smokers' plasma samples. The extraction method utilized in this study provided a high efficiency. The modified methods utilized during this study are applicable

and reliable for the determination of nicotine and cotinine in plasma using HPLC and UV-Visible Spectrophotometer. This method has good results with respect to LOD, LOQ, coefficient of correlation, %R.E. and %RSD.

We advise the other researchers to study the determination of nicotine and cotinine concentrations and their metabolisms in serum and urine samples to complete our study.

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References

Alkhamaisah,S.I., Younes, K.M., Gaipov, A., and Aljofan, M., (2019). "Development and validation of a simple and sensitive HPLC method for the determination of liquid form of therapeutic substances". Electron J. Gen. Med., 16(6):166 – 174.

Bartecchi, C. E., MacKenzie, T. D., and Schrier, R. W., (1995). The global tobacco epidemic. Sci. Am. 5: 44-51.

Benowitz, N. L., (1983). Measurement in the Analysis and Treatment of Smoking Behavior. J. Grabowski and C.S. Bell, Eds. National Institution Drug Abuse Research Monograph 48, U.S. Department of Health and Human Services, Rockland, MD.

Centers for Disease Control and Prevention. (2003). Tobacco Use Among Middle and High School Students—United States, 2002. Morbidity and Mortality Weekly Report, 52(45): 1093–1116. Washington, DC: U.S. Government Printing Office.

Ceppa, F., El Jahiri, Y., Mayaudon, H., Dupuy, O., and Burnat, P., (2000). High-performance liquid chromatographic determination of cotinine in urine in isocratic mode. J. Chromatogr. B 746: 115 - 122.

Davoli, E., Stramare, L., Fanelli, R., Diomede, L., and Salmona, M., (1998). Rapid solid-phase extraction method for automated gas chromatographic - mass spectrometric determination of nicotine in plasma. J. Chromatogr. B 707: 312 - 316.

Doctor, P.B., Gokani, V.N., Kulkarni, P.K., Parikh, J.R., and Sayed, H.N., (2004). Determination of nicotine and cotinine harvesters' urine by solid-phase extraction and liquid chromatography. J. Chromatogr. B 802: 323 - 328.

Elmanfe, G., Omar, S., and Eznien, A. (2020). Determination of Nicotine in Libyan Smokers' and Nonsmokers' Urine samples by Ultraviolet-Visible Spectrophotometry. Journal of Pure and Applied Sciences, 19(5): 126 – 131.

Elmanfe, G., Omar, S., Abdolla, N., and Hassan, A. (2019). Determination of Nicotine in Libyan Smokers' Urine Compared with that of Nonsmokers using Reversed Phase-High Performance Liquid Chromatography (RP-HPLC). Al-Mukhtar Journal of Sciences, 34(3): 172 – 180.

Elmanfe, G. and Abdulla, A. (2014). Spectrophotometric Determination of Nicotine in Some Libyan Smokers' Blood and Compared with Nonsmokers. Paper presented at the 1st Conference on Chemical Sciences and its Applications, 21-23 April 2014, Elbaida-Libya.

Hamm, J. G., (1982). The Handbook of Pest Control; Frederick Fell Publishers Inc.: New York.

Heavner, D.L., Richardson, J.D., Morgan, W.T., and Ogend, M.W., (2005). Validation and application of a method for the determination of nicotine and five major metabolites in smokers' urine by solid-phase extraction and liquid chromatography-tandem mass spectrometry. Biomed. Chromatogr. 19(4): 312 - 328.

Jones, F. E., (1994). Toxic Organic Vapors in the Work Place; Lewis Publishers: London, CRC Press Inc.: New York.

Gupta, R., Prakash, H., Gupta, V.P., and Gupta, K.D., (1997). Prevalence and determinants of coronary health disease in a rural population in India. J Clinl Epidemiol, 50: 203- 209.

Massadeh, A.M., Gharaibeh, A.A., and Omari, K.W., (2009). A Single-Step Extraction Method for the Determination of Nicotine and Cotinine in Jordanian

Smokers' Blood and Urine Samples by RP-HPLC and GC-MS. Journal of Chromatographic Science, 47 : 170 - 177.

Nakajima, M., Yamamoto, T., Kuroiwa, Y., and Yokoi, T.,(2000). Improved highly sensitive method for determination of nicotine and cotinine in human plasma by high-performance liquid chromatography. J. Chromatogr. B 742(1): 211 - 215.

Oddoze, C., Pauli, A. M., and Pastor, J., (1998). Rapid and sensitive highperformance liquid chromatographic determination of nicotine and cotinine in nonsmoker human and rat urines. J. Chromatogr. B 708: 95 - 101.

Orlando, M., Tucker, J.S., Ellickson, P.L., and Klein, D.J. (2004). Developmental Trajectories of Cigarette Smoking and Their Correlates from Early Adolescence to Young Adulthood. Journal of Consulting and Clinical Psychology, 72(3): .400– 410

Padmavati, S., (2002). Prevention of heart disease in India in the 21st century. Need for a concerted effort. Indian Heart J. 54: 99 - 102.

Pasupathi, P., Bakthavathsalam G., Rao Y. Y., and Farook, J., (2009). Cigarette smoking—Effect of metabolic health risk: A review, Diabetes & Metabolic Syndrome: Clinical Research & Reviews 3 : 120 -127.

Pereira, F.J., Rodríguez-Cordero, A., López, R., Robles, L.C., Aller, A.J., (2021). " Development and Validation of an RP-HPLC-PDA Method for Determination of Paracetamol, Caffeine and Tramadol Hydrochloride in Pharmaceutical Formulations". Pharmaceuticals, 14: 466 - 479.

Rani, M., Bonu, S., Jha, P., Nguyen, S.N., and Jamjoum, L., (2003). Tobacco use in India: prevalence and predictors of smoking and chewing in a national cross sectional household survey. Tobacco Control, 12:e4- e4. http://www.tobaccocontrol.com/cgi/content/full/12/4/e4.

Rodricks, J. V., (1992). Calculated Risks; Cambridge University Press: Cambridge, pp 251- 256.

Shin, H.S., Kim J.G., Shin, Y.J., and Jee, S.H., (2002). Sensitive and simple method for the determination of nicotine and cotinine in human urine, plasma and saliva by gas chromatography–mass spectrometry. J. Chromatogr. B 769: 177 - 183.

Silverstein, P., (1992). Smoking and wound healing. Am. J. Med. 93: 225 - 245.

Tollison, R.D., (1988). Clearing the Air : Perspectives on Environmental Tobacco Smoke. Lexington Books, Lexington, MA, U.S.A.

Torano, J.S., and Van Kan, H.J., (2003). Simultaneous determination of the tobacco smoke uptake parameters nicotine, cotinine and thiogyanate in urine, saliva and hair, using gas status of recently exposed subjects. Analyst, 128(7): 838 - 843.

Tyrpien, K., Janoszka, B., and Bodzek, D., (2000). Application of planar chromatography to the nicotine metabolites. J. Chromatogr. A 870: 29 - 38.

Watson, R., and Witten, M., (2001). Environmental Tobacco Smoke., Boca Raton London New York Washington, D.C.: CRC Press.

Wills, T.A., and Cleary, S.D., (1997). The Validity of Self- Reports of Smoking: Analyses by Race/Ethnicity in a School Sample of Urban Adolescents. American Journal of Public Health, 87(1): 56–61.

Wu, C., Siems, W.F., Hill, H.H., and Hannan, R.M., (1998). Analytical determination of nicotine in tobacco by supercritical fluid chromatography-ion mobility detection. Journal of Chromatography A, 811(1-2): 157-161.

Xu, A.S., Peng, L.L., Havel, J.A., Petersen M.E., Fiene J.A., and Hulse, J.D., (1996). Determination of nicotine and cotinine in human plasma by liquid chromatography–tandem mass spectrometry with atmospheric-pressure chemical ionization interface. J. Chromatogr. B 682: 249 - 257.

Zevin, S., Jacob, P., and Neal Benowitz, N., (1997). Cotinine effects on nicotine metabolism. Clinical Pharmacology & Therapeutics, 61(6) : 649-654.