Journal of Chromatography A, 1217 (2010) 307–311

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Determination of pyridine, 2-picoline, 4-picoline and quinoline from mainstream cigarette smoke by solid-phase extraction liquid chromatography/electrospray ionization tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 15 July 2009
Received in revised form 3 November 2009
Accepted 17 November 2009
Available online 24 November 2009

Keywords:
Pyridine
Picoline
Quinoline
Cigarette smoke
2R4F
LC/MS/MS

ABSTRACT

The present paper describes the development and validation of a new reversed-phase liquid chromatography–electrospray ionization tandem mass spectrometric method (RP-HPLC–ESI-MS/MS) for simultaneous determination of pyridine, 2-picoline, 4-picoline and quinoline from mainstream cigarette smoke. Liquid–liquid extraction followed by solid-phase extraction was applied to extract the target analytes from cigarette smoke. Baseline chromatographic separation was achieved by utilizing a Zorbax SB-Aq (4.6 × 150 mm, 5 μm) column in gradient chromatographic conditions with acetonitrile and ammonium acetate buffer as mobile phases. Popular commercially available Indian brand filtered and non-filtered cigarettes were analyzed using the same method. The identification of each chemical was established by chromatographic retention times, analyte specific fragmentation patterns and relative peak area ratios of two product/precursor ion pairs. The limit of detection of this method ranged from 1.74 to 14.32 ng/cig using an injection volume of 20 μl. The reproducibility of this method is excellent and better standard deviations were obtained compared to literature reported values for these chemicals. RSD value is less than 9% for all analytes.

1. Introduction

The progress of technology has brought about more sensitive and reliable modern analytical methodologies that have replaced older ones. In recent years liquid chromatography–tandem mass spectrometry (LC/MS/MS) has gained a very important role in the development of environmental and toxicological research owing to its soft ionization technique, high sensitivity and analyte specific detection based on both retention time and structurally specific analyte fragmentation information. In addition with LC/MS/MS, the use of solid-phase extraction (SPE) for trace level analyte quantitation from complex environmental matrices is predominantly increasing due to more efficient separation of interferences from analytes, reduced organic solvent consumption and easier collection of the total analyte fraction.

Pyridine and substituted pyridines play an important role in the sensory properties of cigarette smoke [1]. These chemicals are important industrial starting materials with an unpleasant smell and high toxicity. Pyridine is widely used as a solvent and intermediate in the production of piperidine, agricultural chemicals, drugs, dyestuffs, paints, rubber products, polycarbonate resins and textile water-repellents, as well as in laboratories. It is frequently found in indoor air and in volatile components of certain foodstuffs [2].

These toxic compounds are well absorbed in the gastrointestinal tract of mammals and undergo extensive metabolism by C- and N-oxidation and N-methylation. Acute pyridine intoxication affects the central nervous system in the human body [2]. Pyridine, substituted pyridines and quinoline also possess adverse human health effects on respiratory organs and reproduction [3–5]. According to the American Lung Association, 20–30% of low-weight babies, up to 14% of preterm deliveries and about 10% of all infant mortality are due to smoking during pregnancy [6], so measurement of known toxins in tobacco smoke is important.

The concomitant presence of high levels of nicotine and relatively low levels of selected volatile compounds in cigarette smoke, along with numerous other chemicals, imparts a significant challenge for development of rapid, sensitive and accurate quantitative methods for the analysis of individual volatile compounds. Literature cited methods used for detection and quantification of pyridines include electrostatic precipitation [7], gas chromatography (GC) [8,9], gas chromatography–mass spectrometry (GC–MS) [10–13]. Since these compounds are volatile, collection of cigarette smoke plays an important role in the determination procedure. Solvent trap was found to be a better device for collection of cigarette smoke rather than a Cambridge filter pad in order to reduce the loss of analyte in collected samples.

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doi:10.1016/j.chroma.2009.11.050

Very few reliable methodologies are available for the accurate and sensitive determination of pyridine, 2-picoline, 4-picoline and quinoline, which the present paper deals with. The current methodology mainly focused on a sample preparation technique, which includes liquid–liquid extraction followed by solid-phase extraction, then detection and quantification of each analyte by LC/MS/MS. A LC/MS/MS method (9 min run time) has been developed and validated by the authors for these four analytes in mainstream cigarette smoke using research reference cigarette 2R4F. The proposed approach will be a viable alternative of conventional GC–MS for the determination of these volatile chemicals from mainstream cigarette smoke.

Over the past years, most of the research reports published on the chemical composition of mainstream cigarette smoke dealt with a single product or reference cigarettes. A limited number of studies have been carried out on commercial cigarettes [14–22]. The present paper incorporates a few commercial Indian brand cigarettes for the analysis of these chemicals and comparison of the levels of these four chemicals with some literature reported commercial US brand cigarettes.

2. Experimental

2.1. Reagents and materials

Both acetonitrile and methanol were HPLC grade and purchased from Spectrochem (Mumbai, India). The ammonium hydroxide and ammonium acetate used were of analytical reagent grade and were obtained from Merck (Mumbai, India). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Methylene Chloride (HPLC grade) was obtained from SRL (Mumbai, India). Hydrochloric acid used here was of analytical reagent grade and supplied by Ranbaxy Fine Chemicals (New Delhi, India). Solid-phase extraction (SPE) cartridges were from Waters (Milford, MA, USA).

Pyridine, 2-picoline, 4-picoline and quinoline (all of analytical standard) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Kentucky reference cigarette 2R4F was procured from Kentucky Tobacco Research and Development Center, University of Kentucky (Lexington, KY, USA). Popular commercially available Indian brand cigarettes were collected from the Indian market.

2.2. Sample preparation

Stock solutions (1 mg/ml) of each of the four analytes were prepared in methanol separately and the corresponding standard solutions for calibration were prepared by proper dilution with methanol.

For preparation of cigarette sample, 10 cigarettes were smoked in a puff volume of 35 ml with 2 s puff duration in every 60 s [23]. Before smoking, the cigarettes were conditioned at 22 °C and 60% humidity for 24 h. The cigarette smoke was passed through two traps containing 25 ml HPLC grade water with 5% hydrochloric acid. After completion of the smoking, both the volumes of water were combined in a 250 ml separatory funnel and 25 ml methylene chloride was added to that. The mixture was vigorously shaken for 10 min and allowed the phases to separate. The organic phase was discarded and upper aqueous phase was transferred to a clean test tube. 2 ml of the aqueous extract was loaded on a Waters Oasis MCX solid-phase cartridge. After acidic wash with 1% hydrochloric acid in methanol, the desired analytes were eluted from the MCX cartridge by using 2 ml of 5% aqueous ammonia in methanol. The eluant was taken in a 10 ml stoppered bubbler and the bubbler temperature was maintained at 10 °C. Dry nitrogen gas was bubbled through the solution at a rate of 40 ml min^{-1} for 2 min to minimize the amount of excess ammonia. Then the final volume of the solution was made 2 ml by addition of methanol and it was transferred to sample vial for LC/MS/MS analysis.

Table 1
Composition, flow rate and timing of the gradient applied for reverse phase separation of the main stream cigarette smoke for pyridine, 2-picoline, 4-picoline and quinoline.

Time (min)	Solvent A (%)	Solvent B (%)	Flow rate (ml/min)	Curve value
0.0	95.0	5.0	1.0	1
0.5	95.0	5.0	1.0	1
5.5	5.0	95.0	1.0	6
6.5	5.0	95.0	1.0	6
7.2	95.0	5.0	1.0	1
9.0	95.0	5.0	1.0	1

Solvent A was 0.01 M ammonium acetate buffer and solvent B was acetonitrile.
Curve value: This sets the rate at which the solvent is to change to the new proportions.
Curve value 1: Immediately goes to specified conditions.
Curve value 6: Linear.

2.3. Instrumental conditions

The chromatographic separation was achieved using a Waters Alliance 2695 Separations Module equipped with built-in auto samplers (Waters, Milford, MA, USA) with a 5.0 μm reverse phase Zorbax SB-Aq (4.6 × 150 mm) column [Agilent Technologies, USA]. The mobile phase solvents A and B were 0.01 M ammonium acetate buffer and acetonitrile respectively and the column temperature was maintained at 30 °C with a flow rate of 1.0 ml min^{-1} in gradient conditions of 9 min run time. A post column flow splitting device was incorporated to deliver the column effluent into the electrospray source of the triple-quadrupole mass spectrometer at approximately 0.2 ml min^{-1} [polyether ether ketone (PEEK) zero dead volume T-piece]. The sample injection volume was 20 μl and two solutions of acetonitrile: water (4:1) and (1:4) were used for washing of the needle and the seal respectively. The auto sampler temperature was maintained at 10 °C. The mobile phase gradient is summarized in Table 1.

Mass spectrometric analyses were carried out on a Quattro micro API triple-quadrupole mass spectrometer (Micromass, Manchester, UK) fitted with a Z-spray electrospray interface in positive ion mode. Instrument control and data acquisition were done with Waters MassLynx software (version 4.0 SCN 509) and processed with Waters QuanLynx Application Manager. A nitrogen generator (Peak Scientific, Renfrewshire, Scotland) was incorporated to supply nitrogen as the cone and desolvation gas at a flow rate of 50 and 250 l h^{-1} respectively. Direct infusion of analyte for optimization procedure was achieved using a Hamilton 1725RNR 250 μl syringe (Hamilton, Reno, NV, USA) at a flow rate

Table 2
Multiple reaction monitoring analysis of pyridine, 2-picoline, 4-picoline and quinoline.

Analyte	Molecular Weight	Precursor ions	Product ions	CE (eV)[a]	CV (V)[b]
Pyridine	79	80	53[c]	24	35
			27[d]	31	38
2-Picoline	93	94	78[c]	25	28
			53[d]	31	35
4-Picoline	93	94	79[c]	28	31
			52[d]	37	37
Quinoline	129	130	103[c]	25	33
			77[d]	32	40

[a] CE, collision energy.
[b] CV, cone voltage.
[c] Quantitation ion.
[d] Confirmation ion.

Table 3
The optimized MS Tune page parameters for the detection of four analytes.

Capillary	3.0 kV
Extractor	3.0 V
RF lens	0.2 V
Source temperature	100 °C
Desolvation temperature	300 °C
LM resolution 1	15.0
HM resolution 1	15.0
Ion energy 1	0.3
Entrance	2.0
Exit	2.0
LM resolution 2	12.4
HM resolution 2	8.8
Ion energy 2	1.0
Multiplier	650

10 μl min^{-1} into the mass analyzer. Following the selection of precursor ions by the first quadrupole mass analyzer, collision-induced dissociation (CID) was carried out using 3.71×10^{-3} mbar of argon (>99.99% pure, Ellenbarrie Industrial Gases Ltd., Kalyani, India) in the hexapole collision cell at laboratory collision energies in the range 10–60 eV. Product ions mass spectra were obtained at a series of collision energies so as to characterize each compounds fragmentation behavior. The optimum collision energy was determined and set for each analyte (Table 2). The mass spectrometer was operated in a 4-channel multiple reaction monitoring (MRM) mode with a dwell time of 0.5 s per ion pair and inter channel delay was 0.01 s. The other mass spectrometric conditions are summarized in Table 3.

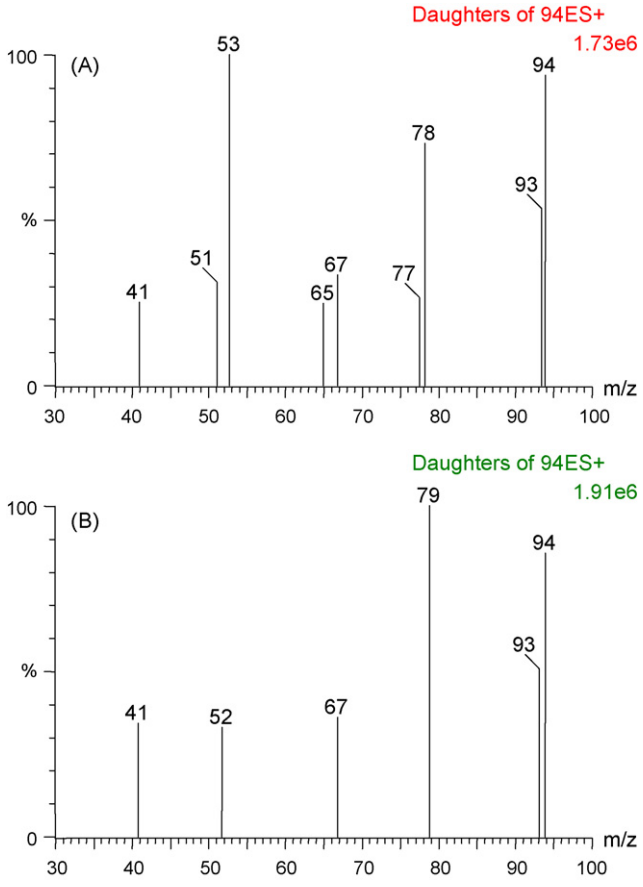

Fig. 1. Typical fragmentation pattern of protonated molecular ion of (A) 2-picoline and (B) 4-picoline are presented here in quantitation experimental condition using centroid mode.

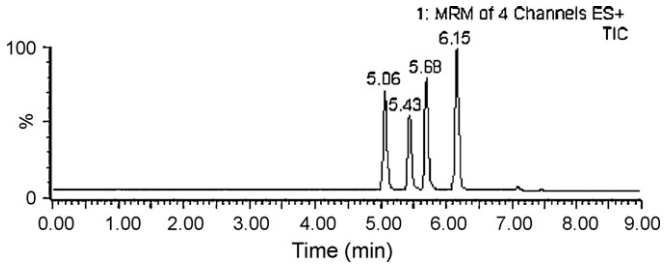

Fig. 2. Typical chromatographic separations of a standard mixture of pyridine, 2-picoline, 4-picoline and quinoline (using 100 ng/ml for each) are shown using tandem mass spectrometric detection. (a) Pyridine (retention time, t_R = 5.06 min). (b) 2-Picoline (t_R = 5.43 min). (c) 4-Picoline (t_R = 5.68 min). (d) Quinoline (t_R = 6.15 min).

3. Results and discussion

3.1. Method development

As all the analytes were nitrogenous compounds and they showed good sensitivity in positive ion ESI; thus, the positive mode was used for further work. Under such conditions, all target analytes yielded mass spectra dominated by the protonated molecular ion. For the collection of cigarette smoke, the solvent trap method was used here instead of pad due to the volatility of the targeted analytes. The initial target was to develop and optimize a liquid–liquid extraction technique as described in the previous study on aromatic amines carried out by the authors [24], but due to volatility of all analytes very little recovery was achieved which compelled the authors to develop the SPE technique. With the combination of liquid–liquid extraction and solid-phase extraction technique, the interferences and matrix effects were minimized, higher recovery was obtained and the targeted analytes were clearly determined by retention time and compound specific fragmentation pattern. The analytes 2-picoline and 4-picoline showed the same protonated molecular ion peak but they were clearly separated by relative retention time and compound specific fragmentation pattern. For 2-picoline the MRM transition was monitored 94 > 78 where as for 4-picoline 94 > 79. The fragmentation pattern for protonated ions of 4-picoline and 2-picoline in the experimental condition are shown in Fig. 1. In the fragmentation pattern of 2-picoline, fragment mass *m*/*z* 78 is present instead of *m*/*z* 79. From this data, it can be assumed that ortho-effect [25] predominates in this fragmentation where as 4-picoline does not contain ortho substitute, so the fragment peak appeared only at *m*/*z* 79.

Among the several solvent combinations tested for optimal resolution and sensitivity of target analytes in LC/MS/MS system, acetonitrile and 0.01 M ammonium acetate buffer was selected as the mobile phase. The best separation of the most challenging regiomers 2-picoline and 4-picoline was achieved by using

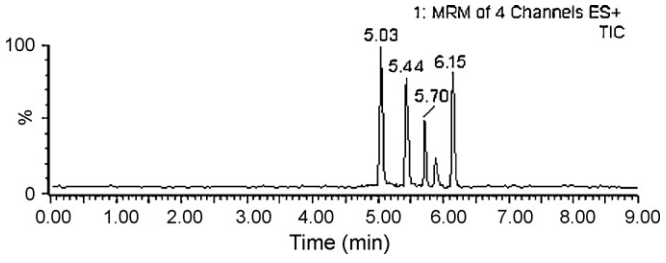

Fig. 3. Typical chromatographic separations of pyridine, 2-picoline, 4-picoline and quinoline from 2R4F cigarette smoke are shown using tandem mass spectrometric detection. (a) Pyridine (retention time, t_R = 5.03 min). (b) 2-Picoline (t_R = 5.44 min). (c) 4-Picoline (t_R = 5.70 min). (d) Quinoline (t_R = 6.15 min).

Table 4
Measurements of recovery for analytes in 2R4F cigarette smoke ($n = 3$).

Analyte	Concentration of standard spiked (ng/ml)	Volume of standard spiked before sample preparation (ml) (in 50 ml)	Volume of standard spiked after sample preparation (ml) (in 2 ml)	Recovery (%)
Pyridine	500	5.0	0.2	85.07
	1000	5.0	0.2	90.15
2-Picoline	200	5.0	0.2	93.16
	500	5.0	0.2	95.28
4-Picoline	50	5.0	0.2	92.67
	200	5.0	0.2	93.11
Quinoline	50	5.0	0.2	84.50
	200	5.0	0.2	87.76

Table 5
Levels of pyridine, 2-picoline, 4-picoline and quinoline in different brands of cigarettes ($n = 5$).

Analyte	2R4F reference cigarette (μg/cig)	RSD (%)	69 mm Indian cigarette with filter (μg/cig)	RSD (%)	84 mm Indian cigarette with filter (μg/cig)	RSD (%)	59 mm Indian cigarette without filter (μg/cig)	RSD (%)
Pyridine	8.65	5.46	3.29	4.83	3.81	3.65	3.41	7.29
2-Picoline	2.14	3.70	0.98	4.87	1.12	4.59	0.93	5.13
4-Picoline	0.84	4.21	0.34	2.51	0.38	2.42	0.39	5.79
Quinoline	0.29	8.39	0.88	3.29	0.95	5.90	1.19	6.89

this mobile phase combination at optimized gradient condition (Table 1). Better sensitivity was achieved using acidic water at lower pH instead of ammonium acetate buffer but resolution decreased in the order pyridine > 2-picoline > 4-picoline and they were eluted as a single peak. So, the resolution and sensitivity were both optimized using ammonium acetate buffer (0.01 M) at a pH 6.6 at 25 °C.

To optimize sensitivity, peak shape and resolution, three different types of HPLC columns viz., Waters Symmetry C18 (4.6 × 50 mm, 3.5 μm), Varian Polaris C18 (4.6 × 50 mm, 5 μm) and Agilent Zorbax SB-Aq (4.6 × 150 mm, 5.0 μm) were evaluated for the separation of these four analytes. After careful evaluation of these columns under selected mobile phase conditions, the present authors successfully optimized the peak shapes and separation using an Agilent Zorbax SB-Aq (4.6 × 150 mm, 5.0 μm) column operated at 30 °C. Baseline chromatographic separation could not be achieved between 2-picoline and 4-picoline by using Symmetry C18 and Polaris C18 column under selected mobile phase condition. Variation of temperature has no effect on separation, resolution or peak shape, so, the work was done in 30 °C column temperature. A typical MRM traces for standard mixtures of these four analytes are shown in Fig. 2 whereas Fig. 3 shows the chromatogram obtained from 2R4F cigarette smoke extract.

3.2. Method validation

A particular feature of the MS–MS detection, which was exploited in these studies, was the capability to differentiate between isomeric analytes, especially those having minor retention differences. Differences in the product ion mass spectra yielded by common precursor ions were decisive in the unambiguous determination of the 2-picoline and 4-picoline. 2-picoline and 4-picoline showed the same protonated molecular ion peak but they were clearly differentiated by their relative retention times, different MRM transitions and relative peak area ratios of two product/precursor ion pairs.

Recovery plays a significant role in method validation procedure for trace level analyte quantitation from complex sample matrix such as cigarette smoke. In this study a spiked recovery method was applied in which standard analyte solutions of two different concentration levels were spiked in 2R4F cigarette smoke extract. For each of the concentration level two sets of solutions were taken. In one set the standards were spiked before work up and analysis was performed after work up. In the other set, the standards were spiked after work up and then analysis was performed. A total of three replicate measurements were performed for each concentration level of each analyte. The method of recovery of each analyte from cigarette smoke was calculated as $Area_b/Area_a$, where $Area_b$ and $Area_a$ are the analytes average peak area obtained from spiking the smoke sample with the standards before and after the sample preparation respectively. Table 4 shows the results of recovery at two different spiked concentration levels. By applying this combined technique sufficiently better recovery was achieved compared to the liquid–liquid extraction technique. More than 85% average recovery was obtained for all the analytes.

The linear calibration curve was obtained for each analyte by plotting the average peak area against the concentration of the analyte. The range of the 8 point calibration curve was varied from 10 to 1000 ng/ml for 2-picoline, 4-picoline, quinoline and 100 to 2000 ng/ml for pyridine. All calibration curves showed excellent linearity with typical correlation coefficient (R^2) between 0.99 and 1.00. The correlation coefficient for pyridine, 2-picoline, 4-picoline and quinoline are 0.9974, 0.9999, 0.9998 and 0.9998 respectively.

The limit of detection (LOD) was calculated as three times of the standard deviation of lowest calibration standard. For LOD determination, lowest calibration standards of all analytes were spiked in

Table 6
Comparison of levels of pyridine, 2-picoline, 4-picoline and quinoline in the mainstream smoke of 2R4F and 1R4F cigarettes.

Analyte	2R4F cigarette (this study) (μg/cig)	2R4F cigarette (Ref. [12]) (μg/cig)	1R4F cigarette (Ref. [12]) (μg/cig)	1R4F cigarette (Ref. [13]) (μg/cig)	1R4F cigarette (Ref. [17]) (μg/cig)
Pyridine	8.65 ± 0.47	7.02 ± 0.70	7.47 ± 0.67	7.82 ± 0.06	8.50 ± 0.60
2-Picoline	2.14 ± 0.08	–	–	2.62 ± 0.06[a]	–
4-Picoline	0.84 ± 0.04	–	–	0.51 ± 0.02	–
Quinoline	0.29 ± 0.02	0.23 ± 0.02	0.30 ± 0.02	–	0.30 ± 0.02

[a] 2 + 3 picoline.

Table 7
Comparison of levels of pyridine, 2-picoline, 4-picoline and quinoline in popular Indian brand cigarettes (in this study) and literature reported major US brand cigarettes (Ref. [17]).

Cigarette/Country	Pyridine (μg/cig)	2-picoline (μg/cig)	4-picoline (μg/cig)	Quinoline (μg/cig)
69 mm cigarette with filter/India	3.29 ± 0.16	0.98 ± 0.05	0.34 ± 0.01	0.88 ± 0.03
84 mm cigarette with filter/India	3.81 ± 0.14	1.12 ± 0.05	0.38 ± 0.01	0.95 ± 0.06
59 mm cigarette without filter/India	3.41 ± 0.25	0.93 ± 0.05	0.39 ± 0.02	1.19 ± 0.08
Marlboro KS F SP/US	13.10 ± 1.10	–	–	0.39 ± 0.03
Parliament 100 F SP Lt/US	3.90 ± 0.60	–	–	0.30 ± 0.02
Merit KS F SP Ult/US	4.80 ± 0.50	–	–	0.16 ± 0.02
Marlboro KS F HPUlt Men/US	3.60 ± 1.10	–	–	0.14 ± 0.02
Virginia Slims 100 F HP Ult Men/US	2.80 ± 0.60	–	–	0.10 ± 0.01
Marlboro 100 F HP Lt/US	7.40 ± 1.00	–	–	0.24 ± 0.03
Merit KS F SP Ultima/US	2.00 ± 0.40	–	–	0.07 ± 0.01

HP: hard pack; SP: soft pack; F: filter; Lt: lights; Ult: ultra lights; KS: king size; Men: menthol.

cigarette smoke extract. Three replicate measurements were performed for each analyte and standard deviations were calculated. Then LOD (ng/cig) for each analyte was calculated using following formula:

$$\text{LOD (ng/cig)} = \frac{[3 \times \text{standard deviation} \times 50\,(\text{Total sample volume})]}{10\,(\text{no. of smoked cigs.})}$$

The LOD value is 14.32, 4.19, 1.74, 3.79 ng/cig for pyridine, 2-picoline, 4-picoline and quinoline respectively. Precision of this method was determined by calculating the relative standard deviations (RSD) of the replicate measurements. All the analytes have RSD values less than 9% (Table 5). The higher RSD of quinoline in 2R4F cigarette smoke compared to the other analytes can be attributed by low content of that in the smoke extract.

The levels of pyridine and quinoline in 2R4F research cigarette smoke have been previously reported [12] and the levels are quite consistent by using these different techniques. A comparison between a few literatures reported values for 2R4F and 1R4F for these four chemicals with values obtained for 2R4F in this study has been presented in Table 6. The levels of each of the analyte in this work were calculated by using the following formula:

$$\text{Analyte (ng/cig)} = \frac{[\text{concentration obtained from calibration curve (ng/ml)} \times 50\,\text{ml (total sample volume]}}{10\,(\text{no. of smoked cigs.})}$$

The values were then corrected by average recovery of each analyte and converted to μg/cig.

Using the same technique three popular Indian brand cigarettes of 69 mm, 84 mm and 59 mm length were also analyzed for these four chemicals and the results are presented in Table 5. The first two brands have filter whereas the third one has no filter. A comparison between these commercial Indian cigarettes analyzed in this study with values for some major commercial US brand cigarettes obtained by Counts et al. [17] is presented in Table 7. It was observed that all commercial Indian cigarettes contain very high levels of quinoline compared to commercial US cigarettes as well as 2R4F reference cigarette. One of the reasons for this variation may be the nature and quality of tobacco crop used in Indian cigarettes which differs from that used in US cigarettes. The difference is probably due to the different soil property, weather and the fertilizer used during its cultivation.

4. Conclusion

The LC/MS/MS method described facilitates fast, quantitative and qualitative determination of pyridine, 2-picoline, 4-picoline and quinoline from the mainstream cigarette smoke extract. The LC run time is only 9 min for these four analytes including re-equilibration of the column. The resolving power of the tandem mass spectrometry provides adequate sensitivity and selectivity for trace level quantitation and confirmation of these four analytes from cigarette smoke and minimizes the interferences of co-extracted materials and background ions. Efficiency of this method for volatiles increased in terms of recovery by the combination of liquid–liquid and solid-phase extraction followed by LC/MS/MS. The developed method was validated and demonstrated for optimum recovery, lower detection limits, accuracy, precision and linearity. Three popular commercially available Indian brand cigarettes were analyzed along with the 2R4F reference cigarette by using this validated method. This LC/MS/MS method of determination of these four analytes may also be used for the determination of these chemicals in other sources where they are present in trace quantities.

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