

Detection of arecoline by simple high-performance thin-layer chromatographic method in Indian nontobacco pan masala

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ABSTRACT

Chewing the habit of blended pan masala containing areca nut with or without tobacco is a common practice in the Indian subcontinent. Arecoline, a pyridine alkaloid presence in areca nut alarmed for oral carcinogenesis and strictly prohibited in the western world. However, in India using blended pan masala is very popular among young and old individuals. In this context, we aimed to detect arecoline in Indian blended nontobacco pan masala sold in Kolkata using a simple densitometric high-performance thin-layer chromatographic (HPTLC) method and for alarming their use in common people. Eleven popularly Indian blended nontobacco pan masala were collected from the territory of Kolkata and isolated arecoline, following solvent extraction method derived for pyridine alkaloid. The quantitative analysis of arecoline was measured using automated software-based HPTLC instruments and validated the method according to International Conference on Harmonization guidelines. Arecoline was detected in all 11 blended nontobacco pan masala samples in a range of minimum 130 to maximum 415 µg/g dry samples. Arecoline is hazardous carcinogenic compound, so the use of Indian blended nontobacco pan masala should be restricted. Further, the method was found suitable for routine quantitative analysis of arecoline in areca nut containing substances.

Key words: Alkaloid, areca nut, arecoline, high-performance thin-layer chromatographic, pan masala

INTRODUCTION

The habit of chewing pan masala with tobacco or without tobacco has long been practice in India and in other South Asian countries such as Myanmar, Thailand, Indonesia, Laos, Cambodia, Vietnam, Papua New Guinea, Taiwan, China, and large number of Islands in Pacific Oceans.^[1] It is also used by the migrant

populations from the Indian subcontinent in parts of Europe, Africa, and Americas.^[2] Now it is estimated that over 600 million individuals are habitual consumers of betel quid with areca nut (*Areca catechu* L.) in one form or the other worldwide.^[3] Last few decades, the ill effects of uses of chewing tobacco, betel quid, and also areca nut have been focused by World Health Organization (WHO) and other scientific institutions.^[4-6] The major active constituent present in areca nut has marked by arecoline (1,2,4,5,-tetrahydro-1-methylpyridine carboxylic acid, molecular weight 155.19 Da), a pyridine alkaloid.^[7] Arecoline in oral cavity undergo nitrosation and give rise of N-nitrosamines, a known carcinogen.^[4] The

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development of oral submucous fibrosis, a precancerous state, is universal among areca nut user.^[8-10] The carcinogenic potentiality, as well as its cell transformation ability, has already been reported.^[11-14] In Kolkata, blended pan masala is very popular among young and old individuals and now raising the question of their use.^[15] Though the use, making or trading pan masala is strictly prohibited in some parts of India, others are still not a concern. Till now, there is no limit value or guidelines of use of arecoline presence in areca nut. Perhaps, there is no simple method of its isolation and detection. In this regard, we pointed out the use of this harmful ingredient without any restriction and also tried to develop and validate a simple detection method of arecoline estimation from areca nut content in blended nontobacco pan masala samples freely available in Kolkata.

MATERIALS AND METHODS

Test samples

Eleven nontobacco branded pan masala popularly marketed in Kolkata and cutting areca nut (normal) were purchased from the local shop [Table 1]. The samples were coded as TS-1 to TS-12 for blind analysis.

Extraction of arecoline

One gram powdered of each test sample was mixed with 10 ml of methanol, occasionally shaken, and kept at room temperature (22–25°C) for 24 h. In the following day, the suspension was filtered, and the sample was washed with an adequate amount of methanol. The filtrate was made to the final volume at 10 ml. Thereafter, the solvent was evaporated under vacuum. The dried extract was dissolved in 2 ml of 0.1 N HCl. Then the solution was washed with 3 ml of chloroform. One milliliter of the acid layer was taken and neutralized with 1 N ammonium hydroxide. Thereafter, 10 ml chloroform was added and shaken well. The chloroform layer was taken and saturated with anhydrous sodium sulfite. The chloroform layer was carefully taken

and evaporated it to dryness. Finally, the eluted material was dissolved in HPLC grade acidic methanol (methanol: HCl = 98:2 v/v).^[16,17]

Standard calibration

Arecoline HBr (Sigma-Aldrich Laborchemikalien, GmbH; trade name Vertranal; Product No. 31593, Batch No. SZBB103XV, molar mass 236.11 g/mol) was dissolved in acidic methanol at the concentration of 1 mg/ml and applied as 300 ng, 700 ng, 1.1 µg, and 1.5 µg to prepare the standard calibration curve by plotting peak areas versus concentration. The accuracy of method has been verified.

High-performance thin-layer chromatographic analysis

The test samples (TS-1 to TS-12) were spotted in the form of bands with Camag microliter syringe on a precoated silica gel plates (Merck, 60F₂₅₄, 20 cm × 20 cm) using Camag Linomat 5 applicator. The plates developed in a solvent system (chloroform: Toluene: Ethyl acetate: Diethylamine = 7:7:4:1) in Camag glass chamber for 30 min and air dried for 5 min at 60°C. The densitometric scanning were performed on Camag TLC Scanner 3 at absorbance 220 nm (D2 lamp) and operated by multilevel win CATS planar chromatography manager [Table 2].^[18,19] The amount of arecoline present in the samples was calculated against respective markers and expressed per gram of dry pan masala. The experiments repeatedly confirmed the results.

Method validation

International Conference on Harmonization (ICH) guidelines were followed for the method validation of the analytical procedures.^[20] The method was validated for precision, repeatability, and accuracy. The repeatability of the method was checked by repeated scanning of the same spot of arecoline (1 µg), 6 times and was expressed as coefficient of variance (%CV). The variability of the method was studied by analyzing aliquots of arecoline (as 300 ng, 700 ng, 1.1 µg, and 1.5 µg) on the same day and on different days and the outcome data were expressed as %CV. The recovery studies

Table 1: Concentration of arecoline on Indian blended nontobacco pan masala

Sample number	Sample name and manufacture name	Arecoline (ng/mg sample)
TS-1	Rajnigandha Flavored Pan Masala (DS Pvt. Ltd., Guwahati, Assam, India)	376.9±7.71
TS-2	Parag Pan Masala (Pan Parag India Ltd., Vadodara, Gujarat, India)	362.5±4.63
TS-3	Paras Premium Pan Masala (Paras Surti Products Pvt. Ltd., Kolkata, West Bengal, India)	349.2±6.36
TS-4	Dilruba Sahi Pan Masala (Ashok and Co. Pan Bahar Ltd., New Delhi, India)	255.2±4.01
TS-5	Pan Parag Pan Masala (Pan Parag India Ltd., Vadodara, Gujarat, India)	386.1±2.90
TS-6	Shikhar Pan Masala (Trimurti Fragrances Pvt. Ltd., Kanpur, Uttar Pradesh, India)	415.6±3.83
TS-7	Chutki Mouth Freshener (K.K. Sales, Delhi, India)	132.7±5.92
TS-8	Tiranga Pan Masala (Kays Fragrances Pvt. Ltd., Bahalgarh, Haryana, India)	384.7±2.65
TS-9	Sir Gold Finest Pan Masala (Prabhu Aastha Enterprise (P) Ltd., Kanpur, Uttar Pradesh, India)	294.9±4.06
TS-10	Bahar Heritage Pan Masala (Ashok and Co. Pan Bahar Ltd., New Delhi, India)	322.7±5.94
TS-11	Sweety Supari Kesar Scented (Shri Girraj Supari Traders, Mathura, Uttar Pradesh, India)	167.4±3.55
TS-12	Areca nut (normal control)	434.4±4.42

All tests were conducted 6 times; values were depicted as mean±SD. SD: Standard deviation

Table 2: HPTLC condition for arecoline estimation

Instrument	CAMAG Linomat 5
Detector	CAMAG TLC Scanner 3
Development	Twin Trough Chamber
Calibration mode	Multilevel
Evaluation mode	Peak height and area
Plate	Silica gel 60 F254
Plate size	20×20 cm
Mobile phase	Chloroform: toluene: Ethyl acetate: Diethylamine=7:7:4:1
Syringe size	100 μ l
Band length	8 mm
Application volume	4-10 μ l
Lamp	D2
Slit dimensions	4×0.2 μ m
Wavelength	220 nm

HPTLC: High-performance thin-layer chromatographic, TLC: Thin layer chromatographic

were done at three levels (50%, 100%, and 150% addition). The percent recovery and average percent recovery was calculated for studying accuracy of the method.

RESULTS

Figure 1 shows the high-performance thin-layer chromatographic (HPTLC) chromatogram of standard arecoline and its R_f value was found to be at 0.58. The linear regression of standard calibration curve via area was established as $r^2 = 0.99907$ and standard deviation 2.24% [Figure 2]. Minimum detection limit of arecoline was reported as 3.25 ng. Spectral comparison for arecoline of all test samples was matched with known standards at 220 nm [Figures 3 and 4]. All fingerprints of densitometric chromatograms of test samples were represented in Figure 5, while arecoline content was tabulated in Table 1. Normal areca nut (nonblended) showed the presence of a highest amount of arecoline, that is, 434 ng/mg of its dry weight. Moreover, out of 11 blended samples of nontobacco pan masala, Shikhar Pan Masala showed maximum (415 ng/mg), while Chutki Mouth Freshener exhibited minimum (132 ng/mg) amount of arecoline [Table 1]. The ranges of arecoline in blended pan masalas were 132–415 ng/mg dry samples. The amount of arecoline observed in the test samples of pan masala were represented as follows: Shikhar Pan Masala > Pan Parag Pan Masala > Tiranga Pan Masala > Rajnigandha Flavored Pan Masala > Parag Pan Masala > Paras Premium Pan Masala > Bahar Heritage Pan Masala > Sir Gold Finest Pan Masala > Dilruba Sahi Pan Masala > Sweety Supari Kesar Scented > Chutki Mouth Freshener.

DISCUSSION

The use of blended pan masala is gaining social acceptance, and consumption is also increasing with an increase in the

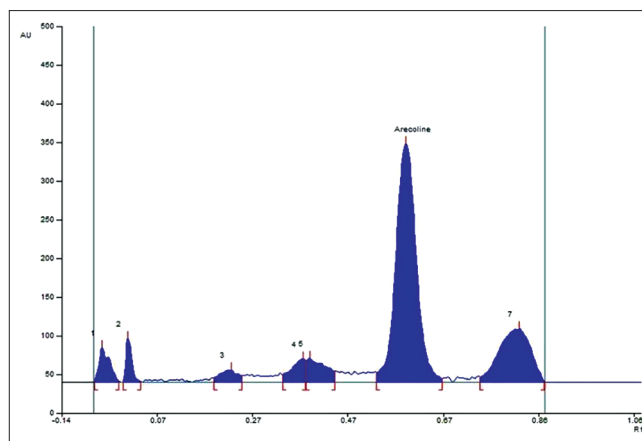


Figure 1: High-performance thin-layer chromatographic fingerprint of standard arecoline

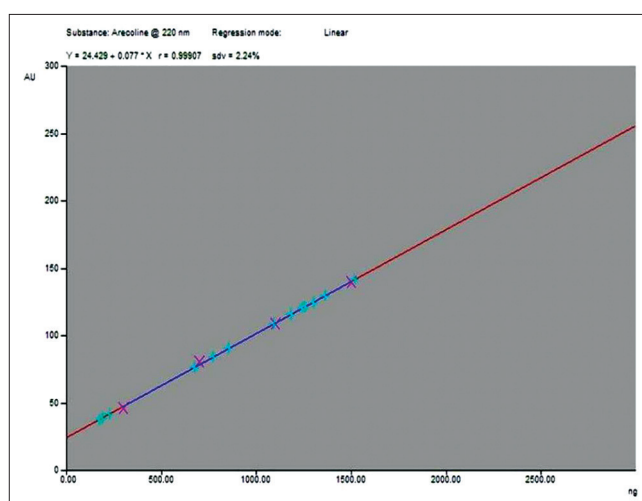


Figure 2: Calibration curve for arecoline

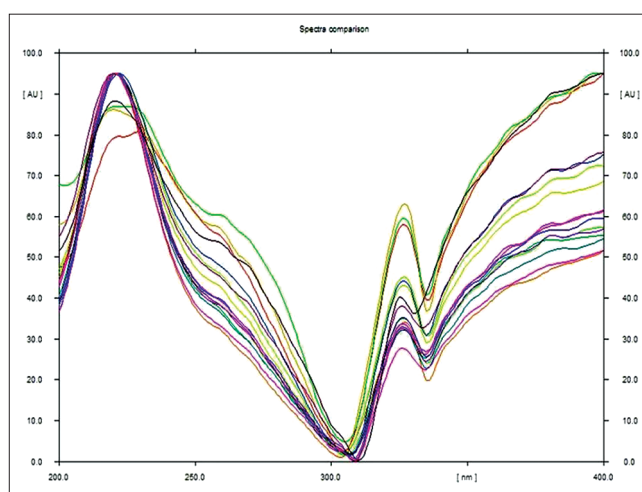


Figure 3: Spectral analysis of arecoline of test samples

sales of pan masala products every day. The WHO and International Agency for Research on Cancer classified areca nut and arecoline as a Group 1 human carcinogen.^[1,4]

Three major N-nitroso compounds of arecoline namely N-nitroso-guvacoline, 3-methyl-nitrosamino propionitrile, and 3-methyl-nitrosoamino propionaldehyde have been

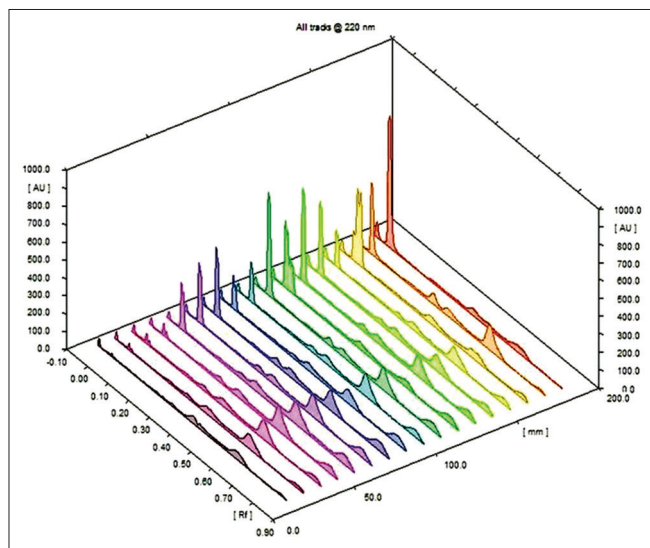


Figure 4: Comparison of high-performance thin-layer chromatographic spectral analysis at 220 nm

identified in areca nut or pan masala chewers.^[4] Genotoxic effects of these nitrosamines have well documented.^[21] Withdrawal symptoms such as mood swings, anxiety, irritability, reduced concentration, sleep disturbance, and craving were found to be associated with trying to quit the habit of pan masala chewing.^[22] Genotoxic reactants formed from arecoline and areca nut derived nitrosamines can lead to DNA- and genetic-damage in exposed oral keratinocytes.^[23-25] Recently, determination and pharmacokinetic studies of arecoline has been conducted in dog.^[26] Earlier studies reported different branded Indian pan masala containing polycyclic hydrocarbons and pesticides.^[27] The present work alarmed that arecoline was abundantly present in popularly branded nontobacco pan masala marketed in Kolkata territory and also pointed out that the habit of chewing pan masala becoming more deleterious for society if not restricted before time. Further, it is also important that amount of arecoline in areca nut varies with seasonal and geographical variations (10–600 ng/mg) and it may be reduced variably following processing of the nut by different methods.^[28,29] In this study, arecoline was separated and identified from eleven commercially blended pan masala and also from normal

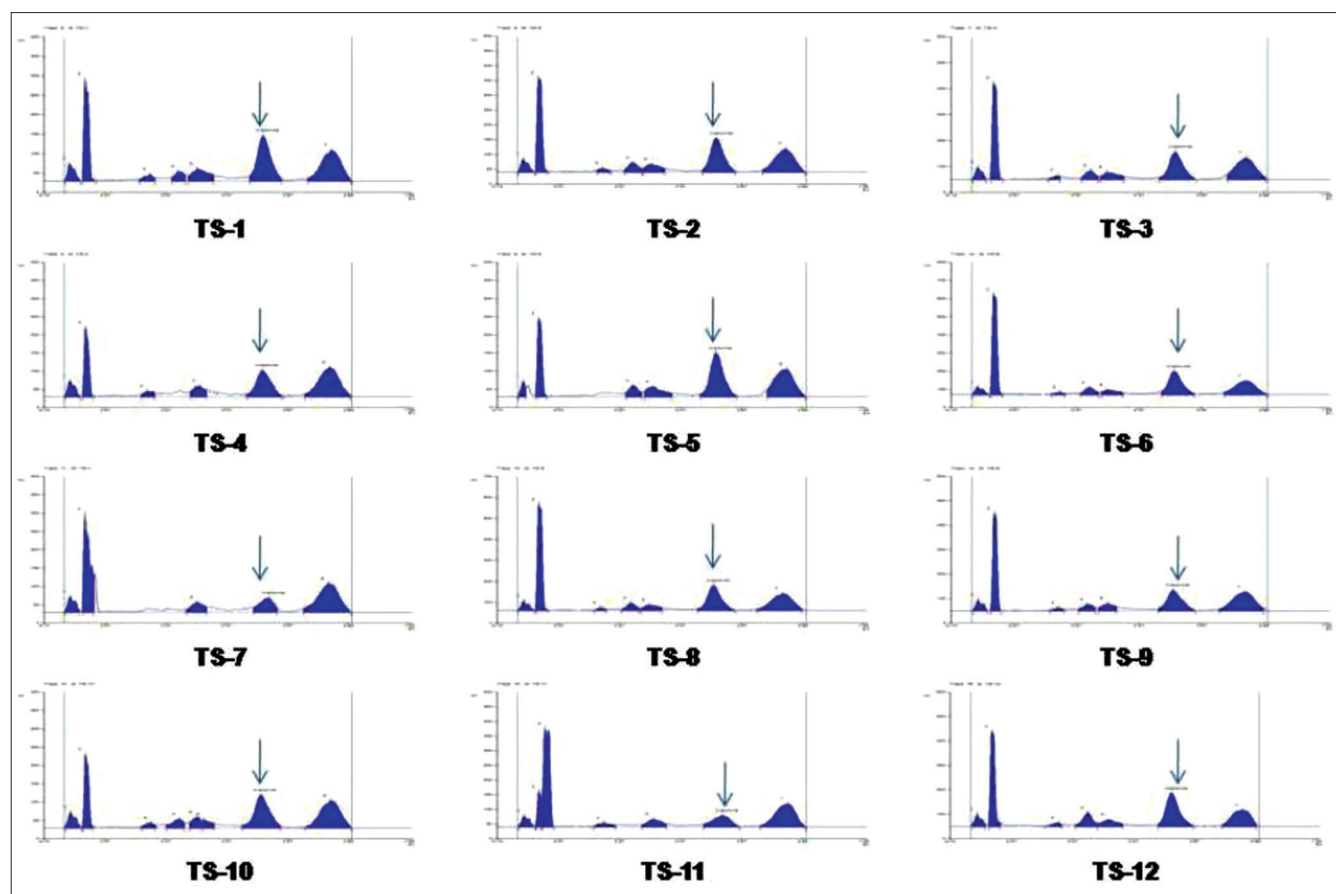


Figure 5: High-performance thin-layer chromatographic fingerprints of blended nontobacco pan masala samples. TS-1: Rajnigandha Flavored Pan Masala; TS-2: Parag Pan Masala; TS-3: Paras Premium Pan Masala; TS-4: Dilruba Sahi Pan Masala; TS-5: Pan Parag Pan Masala; TS-6: Shikhar Pan Masala; TS-7: Chutki Mouth Freshener; TS-8: Tiranga Pan Masala; TS-9: Sir Gold Finest Pan Masala; TS-10: Bahar Heritage Pan Masala; TS-11: Sweety Supari Kesar Scented; TS-12: Control Areca nut; arrow indicated arecoline

areca nut with densitometric HPTLC. The method was validated according to ICH guidelines, and it may be helpful to detect arecoline from areca nut containing preparations. Therefore, this method can be successfully employed for detection of arecoline because of its short analysis time and use of small amounts of reagents as well as for its sensitivity, reproducibility, accuracy, and simplicity.

CONCLUSION

Arecoline is abundantly present in Indian blended nontobacco pan masala and that can be detectable by simple HPTLC method.

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Conflicts of interest

There are no conflicts of interest.

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