

Estimation of arecoline content of various forms of areca nut preparations by high-pressure thin-layer chromatography

Dipti Dutta, C. Ramanna, V. V. Kamath

Department of Oral and Maxillofacial Pathology, Dr. Syamala Reddy Dental College Hospital and Research Centre, Bengaluru, Karnataka, India

Keywords

Arecoline, boiled areca nut, high-pressure thin-layer chromatography, pan masala, raw areca nut, roasted areca nut

Correspondence

C. Ramanna, Department of Oral and Maxillofacial Pathology, Dr. Syamala Reddy Dental College Hospital and Research Centre, Bengaluru - 560 037, Karnataka, India.
E-mail: ramannamdc@gmail.com

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Abstract

Background: Areca nut and its commercial preparations such as pan masala have been causatively linked in the disorder oral submucous fibrosis (OSF). Arecoline, major alkaloid of areca nut, is involved in stimulating fibrosis through transforming growth factor beta pathway. Areca consumed in various forms and with additives alters the arecoline content of the nut.

Aims: Estimation of levels of arecoline in various areca nut preparations consumed in the country (raw, boiled, and roasted) along with commercially prepared sample of areca nut (pan masala).

Materials and Methods: Estimation and validation were performed by high-performance thin-layer chromatography system (CAMAG® Muttenez, Switzerland). Four samples of 5 g each of raw, roasted, boiled, and pan masala containing areca nuts were assessed. Pure arecoline was used for validation as a control. Three runs of each with inter- and intra-day validations were carried out as per established protocols.

Results: Raw areca nut contains the highest concentration of arecoline (1.15 ± 0.008) followed by pan masala preparations (0.94 ± 0.006), least content in boiled areca nut (0.79 ± 0.009), while roasted variety exhibited an intermediate level (0.85 ± 0.007).

Conclusions: Alteration of contents of the areca nut has great implications in its capacity to induce the potentially malignant disorder of OSF. The use of raw areca nuts in commercial preparations and well-known observations of greater incidence of the development of OSF and probable malignant transformation with these forms is probably related to their arecoline content.

Introduction

Oral submucous fibrosis (OSF) is an insidious, chronic disease affecting any part of the oral cavity and sometimes the pharynx. The lesion was first described by Schwartz in 1952. It shows a clear-cut geographical and ethnic predisposition among the Southeast Asian population. The hallmark of the disease is a chronic change in the fibro-elasticity of the lamina propria. Juxta-epithelial inflammatory reaction and an associated epithelial atrophy are the other characteristic features.^[1]

The pathogenesis of the disease is thought to be multifactorial, with chewing of areca nut recognized as one of the most significant risk factors for OSF. Areca nut is perused in many forms in the Indian subcontinent ranging from raw to

commercial varieties. Pan masala, as it is traditionally called, is a popular dispensation among the commercial products. The latter is a concoction of areca nut powder with specified and unspecified additives. When combined with tobacco it is called gutkha. Marketed aggressively and packaged intelligently, the pricing made affordable, its popularity among the youth has led to its widespread use.^[2-6]

Areca nut is the seed of the fruit of the oriental palm *Areca catechu* that is native to Sri Lanka, tropical India, Malaysia, Polynesia, Micronesia, South China, East Indies, the Philippine Islands, parts of East Africa, and most places in the South Pacific Islands.^[2]

The habit of areca nut chewing impinges on the daily lives of about one-tenth of the world's population. It is the 4th most

addictive psychoactive substance consumed. The addictive potential is due to the inhibition of gamma-aminobutyric acid (GABA) uptake in the nervous system by the areca alkaloids.

Areca nut extract contains alkaloids such as arecoline, arecaidine, guvacine, guvacoline, flavonoids such as tannins and catechins, and traces of copper.^[2] The pyridine alkaloids, i.e., arecoline and arecaidine are thought to play a major role in the development of adverse effects resulting from this chewing habit. Arecoline penetrates the oral mucosa and is hydrolyzed into arecaidine, which in turn induces fibroblast proliferation, increased collagen synthesis, and decreased collagen degradation.^[7]

The processing treatments of the fresh nuts change the chemical composition, astringency, and the flavor of the nut. The roasted nut possesses the highest tannin content followed by the raw and the boiled nut. The arecoline content as determined by Awang is highest for the sun-dried raw variety followed by the roasted and the boiled variety.^[8-10]

Previously, few methods have been reported for qualitative and quantification of arecoline content, these include thin-layer chromatography (TLC), ultraviolet (UV) spectrophotometry, gas chromatography (GC), GC-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC), and liquid chromatography-ion trap MS.^[11-20]

The objective of this study was to develop and validate a high-pressure TLC (HPTLC) method for quantification of arecoline and to compare the arecoline content in various forms of areca nut (raw, roasted, and boiled) and pan masala. The HPTLC technique was used as it is a simple, sensitive, rapid, and economical method for qualitative and quantitative analytical tasks.^[21]

Materials and Methods

Extraction of arecoline

About 5 g of raw areca nut powder was moistened with concentrated ammonia and mixed with 50 ml of chloroform in a sonicator for 2 h. The mixture was filtered using Whatman filter paper. 2% sulfuric acid was added to the filtrate and shaken, and the acid layer was retained. This step was repeated three times to ensure maximum extraction of arecoline from chloroform. The arecoline salts extracted in the acid layer were then purified from lipid impurities by repeatedly shaking with petroleum ether and discarding the ether layer. The acid layer was then made basic (pH 10) by adding a few drops of ammonia and extracted again with chloroform three times to ensure complete extraction of arecoline from the acid. The chloroform layer was retained and inorganic ion impurities were removed by adding little distilled water three times and discarding the water layer. The excess water was removed from the chloroform solution by adding anhydrous sodium sulfate. The solution was left undisturbed for 30 min and then filtered. The filtrate was then allowed to evaporate to dryness and 1 ml of 0.1 N HCl added to make a salt solution. The same procedure was followed for roasted, boiled areca nut, and

pan masala powder. The solution was then subjected to HPTLC to quantify the percentage of arecoline in the various forms of areca nut (raw, roasted, and boiled) and pan masala.

HPTLC procedure

Instrumentation

The HPTLC system (CAMAG®, Muttenz, Switzerland) consisted of (i) Linomat 3 sample applicator using 100 µl syringes connected to a nitrogen cylinder, (ii) Chamber ADC 2 containing twin trough chamber 20 cm × 10 cm, and (iii) CAMAG TLC scanner 3 connected to WINCATS software. The columns consisted of normal phase aluminum plates (Merck, Darmstadt, Germany) with aluminum-backed layers' silica gel 60 F₂₅₄ (10 cm × 10 cm, 0.2 mm thick). Before use, plates were washed with methanol and dried for 30 min at 100°C. Filtered solutions were applied with nitrogen flow.

Reagents

The various forms of areca nut were procured from Wayanad district, Kerala. Pan masala was purchased from the local market. Arecoline hydrobromide was used as the standard and was purchased from Sigma-Aldrich (Vetranal, analytical standard).

Standard preparation

About 100 mg of the arecoline hydrobromide powder was accurately weighed and transferred to 10 ml volumetric flask, and the final volume of stock solution was made up to 10 mg/ml with methanol.

Sample preparation

The sample solution was diluted with 9 ml of methanol to get a dilution factor of 1:10.

Mobile phase

Ethyl acetate: methanol:distilled water - 7:17:1. The developing solvents, i.e., the polarity of solvents and ratios were carefully optimized before the analyses.

Validation study

The validation parameters were standardized for pure arecoline followed by analyses of the samples at the standard parameters. The developed HPTLC method was validated according to the International Conference on Harmonization guidelines in terms of specificity, linearity, precision and accuracy, limit of detection (LOD), and limit of quantification (LOQ). For the specificity study, it was carried out to check the absence of interference by the solvent and the degradation products of arecoline. For the linearity study, six levels of concentrations within the range 60-360 ng/spot of pure arecoline were prepared. The linearity was then evaluated by the linear regression analysis. The precision of the assay was determined by repeatability (intraday) and intermediate precision (interday). Repeatability was evaluated by assaying samples, at the same concentration, and during the same day. The intermediate precision was studied by comparing the assays on different days (6 days). For the accuracy study,

it was determined by recovery of known amounts of standard arecoline ($n = 6$). For LOD and LOQ, they were determined on the basis of response and slope of the regression equations.

HPTLC procedure

The operating conditions were syringe delivery speed - 10 s μl^{-1} (100 nL s^{-1}); injection volume - 3 μl ; bandwidth - 8 mm; distance between bands - 4 mm; distance from bottom - 15 mm.

The HPTLC plates were developed in the developing chamber ADC 2. The developing chamber was saturated with the mobile phase ethyl acetate: methanol:distilled water in the ratio of 7:17:1 (v/v/v) for 20 min at room temperature. The length of the chromatogram run was between 70 and 80 mm from the point of application. The developing time was approximately 15-20 min and retention factor was 4.1.

Detection and visualization of the chromatogram was done at an optimized wavelength of 254 and 366 nm. After development, the chromatogram was scanned using a TLC scanner (WINCATS software). The scanner operating parameters were: Mode - absorption/reflection; slit dimension - 5 mm \times 0.1 mm; scanning rate - 20 mm/s; monochromatic bandwidth - 20 nm. The concentration of arecoline was then quantified in each sample by calculating the area under the respective peaks, and the value was expressed as a percentage dry weight of the nut powder.

Results

The calibration curves for arecoline were constructed by plotting concentration versus peak area and showed good linearity in the 60-360 ng/ml range [Table 1]. A representative calibration curve of the average calibration curve data obtained is shown in Figure 1. The representative linear regression equation was $y = 14.69x - 1.466$, with a correlation coefficient of $r^2 = 0.9987$, i.e., highly significant for the method. The LOD was 35 ng/spot and LOQ was 60 ng/spot, respectively, indicating a high sensitivity of the method [Table 2].

Percentage recovery of arecoline was 99.44%, indicating a good accuracy of the method [Table 3]. The precision of the method was determined by repeatability intra-and inter-day. The repeatability was expressed as % relative standard deviation (%RSD) and the value obtained was 0.2805% [Table 4]. The %RSD obtained was lower than 2, indicating good precision.

Previous reports set the detector range for arecoline to be near 230-360 nm. However, for our study, the detection range was set at 254 and 366 nm.

Extraction and quantification of arecoline from various forms of areca nut and pan masala revealed a concentration of 1.15% of arecoline in raw areca nut, 0.95% in pan masala, 0.85% in roasted areca nut, and 0.79% in boiled areca nut [Table 5 and Graph 1].

Discussion

The word "areca" is derived from the Malay word "adakka" or from "adakeya," the Indian equivalent. It is the fourth most

commonly used psychoactive substance, after caffeine, nicotine, and alcohol.^[7]

Areca nut is the seed of the fruit of the oriental palm *A. catechu*. The plant is an unbranching, long plant extending up to 15-30 m.

Table 1: Calibration curve for standard pure arecoline ($n=6$)

Pure arecoline in ng/ml	AUC
60	879
120	1761
180	2645
240	3522
300	4408
360	5285

AUC: Area under curve

Table 2: Method performance/validation parameters for quantification of pure arecoline by proposed HPTLC method

Parameters	Method (pure arecoline)	Acceptance criteria (maximum acceptable)
Selectivity	Selective	
Specificity	Specific	No interference observed
Linear range (ng/spot)	60-360	Linearity, accuracy, and precision were within the range
Correlation coefficient (r^2)	0.9987	Between 0.9 and 1.1
Linear regression equation $y=mx+c$	$y=14.69x-1.466$	
LOD (ng/spot)	35	
LOQ (ng/spot)	60	
% recovery	99.44	Within 90-110%
Repeatability (%RSD, $n=6$)	0.2805%	RSD<2%
Precision (% CV)		%CV<2%
Intraday ($n=6$)	0.54-1.86%	
Interday ($n=6$)	0.56-1.79%	

HPTLC: High-performance thin liquid chromatography, LOD: Limit of detection, LOQ: Limit of quantification, RSD: Relative standard deviation, CV: Coefficient of variance

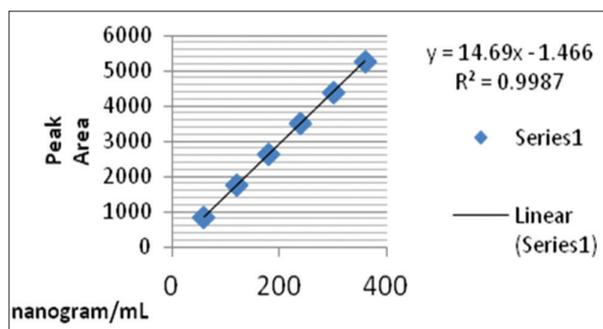


Figure 1: Calibration curve for standard pure arecoline

Table 3: Recovery studies of pure arecoline by spiking

Concentration of standards (ng/spot)		Area spotted		Total area (sample+standard)	Total area obtained	% recovery
Sample content	Spiked amount	Sample area	Spiked area			
100	50	1465	731	2196	2180	99.27
100	100	1465	1458	2923	2907	99.45
100	150	1465	2202	3667	3656	99.61
Average recovery (%)						99.44

Table 4: Intra- and inter-day precision of pure arecoline by HPTLC method ($n=6$)

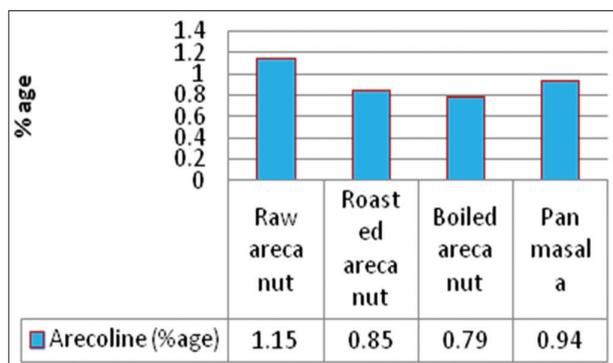
Amount (ng/spot)	Intraday precision		Interday precision	
	SD in area	%CV	SD in area	%CV
60	6.91	0.95	14.92	1.79
120	7.13	0.54	15.27	0.98
180	26.25	1.05	31.63	1.41
240	27.12	0.89	27.92	0.98
300	62.75	1.86	15.38	0.56
360	29.12	0.92	17.22	0.81

HPTLC: High-performance thin liquid chromatography, SD: Standard deviation, CV: Coefficient of variance

Table 5: Arecoline content in different forms of areca nut and pan masala extracts as determined by HPTLC

Name of the areca nut	Arecoline (%)
Raw areca nut	1.15±0.008
Pan masala areca nut	0.94±0.006
Boiled areca nut	0.79±0.009
Roasted areca nut	0.85±0.007

HPTLC: High-performance thin liquid chromatography

**Graph 1:** Concentration of arecoline in various forms of areca nut and pan masala

The plant is native to Sri Lanka, tropical India, Malaysia, Polynesia, Micronesia, South China, East Indies, the Philippine Islands, parts of East Africa, and most places in the South Pacific Islands and bears fruit all year. The fruit is ovoid or oblong with a pointed apex, measuring 3-5 cm in length and 2-4 cm in diameter. The outer surface is green when unripe and orange-yellow when

ripe. The seed (endosperm) is separated from a fibrous pericarp, is rounded with a truncated base, and is opaque and buff-colored with dark wavy lines. It has a characteristic astringent and slightly bitter taste and can be consumed at different stages of maturity according to preference. The nut may be used fresh or it may be dried and cured before use, by sun-drying, baking, boiling, roasting, or covering it with mud to soften the nut for consumption. These treatments change the flavor of the nut and its astringency.^[2]

The major constituents of the areca nut include carbohydrates, fats, proteins, crude fiber, polyphenols (flavonols and tannins), alkaloids, and mineral matter. Polyphenols (flavonols and tannins) constitute a large proportion of the dry weight of the nut and are responsible for the astringent taste of the nut. Alkaloids form the most important biological components and include structurally related pyridine alkaloids, i.e., arecoline, arecaidine, arecaine, arecolidine, guvacine, isoguvacine, guvacoline, and coniine. The polyphenol content of the nut varies depending on the region of cultivation, degree of maturity, and method of processing. It is highest in unripe nuts, the content decreasing with increase in maturity.^[2,8] In a study done by Awang,^[10] the tannin content in the roasted variety of areca nut was found to be 5-41%, 25% in the sun-dried variety, and 17% in the boiled variety.

Wide variations in the arecoline content of areca nut have been demonstrated in commercially available nuts, ranging between 0 and 1.4%. Arecoline content is reduced following processing of the nut. The content is reduced to 1.35% by sun-drying, to 1.29% by roasting, to 0.7% by soaking in water, and to 0.1% by boiling in water as stated by a study done by Awang using HPLC.^[11]

Other traces of elements identified in the nuts include sodium, magnesium, chlorine, calcium, vanadium, manganese, copper, and bromine. The mean concentration of copper in samples of processed, commercially available areca nut is $18 \pm 8.7 \mu\text{g/g}$. The copper content of processed areca nut is 2.5 times that of the raw unprocessed nut.^[2]

Arecoline is a pyridine alkaloid with a molecular formula $\text{C}_8\text{H}_{13}\text{NO}_2$ (molecular mass = 155.1). The systematic name is 1, 2, 5, 6-tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester; methyl 1-methyl-1, 2, 5, 6-tetrahydropyridine-3-carboxylate. It is an oily liquid that is soluble in water, alcohols, chloroform, and ether. The UV detection range for arecoline is 230-360 nm.^[19] Arecoline hydrobromide has a molecular weight 236.11 and empirical formula $\text{C}_8\text{H}_{13}\text{NO}_2\text{HBr}$. The systematic

name is 1-methyl-1, 2, 5, 6-tetrahydro-3-pyridinecarboxylic acid methyl ester hydrobromide.^[22]

Arecoline, the principal alkaloid in areca nut, acts as an agonist primarily at muscarinic acetylcholine receptors, acts as a stimulant of the central and autonomic nervous system, and causes increases in the levels of monoamines such as noradrenaline as well as acetylcholine at higher doses. This leads to subjective effects of increased well-being, alertness, and stamina. Arecaidine may have anxiolytic properties through inhibition of GABA reuptake. The preferred route of administration, chewing, leads to rapid absorption of these alkaloids through the buccal mucosa, leading to an onset of these effects within 5 min, lasting for about 2-3 h. Other reported effects including staving off hunger and a postprandial digestant.^[23] Arecoline also has parasymphathomimetic properties that cause an increase in the salivary flow rate.^[24] Arecoline is genotoxic and cytotoxic both *in vitro* and *in vivo* through oxidative stress-dependent mechanisms.^[2,25]

Arecoline is also said to affect the fibroblasts and keratinocytes leading to depressed DNA synthesis, cell cycle arrest, cell death, both increased and decreased collagen production, and cytokine synthesis. Two concentrations of arecoline appeared to be

critical, being 0.1 µg/ml for collagen stimulation and 10.1 µg/ml for cytotoxicity.^[26]

Arecoline gets metabolized in the liver and kidneys to form metabolites including arecaidine, arecoline N-oxide, arecaidine N-oxide, N-methylnipecotic acid, N-methylnipecotylglycine, arecaidinylglycine, arecaidinylglycerol, arecaidine mercapturic acid, arecoline mercapturic acid, and arecoline N-oxide mercapturic acid. Arecaidine and N-methylnipecotic acid are the major metabolites.^[27]

Previously, few methods have been reported for qualitative and quantification of arecoline content; these include TLC, UV spectrophotometry, GC, GC-MS, and HPLC.

TLC, a form of chromatography, is named so as it uses an inert solid material such as silica gel that serves to support a thin-layer liquid as the stationary phase. HPTLC is an enhanced form of TLC. The enhancements made to the basic method of TLC include automation at different steps, increased resolution, and more accurate quantitative measurements and analysis. Automation is useful to overcome the uncertainty in droplet size and position when sample is applied to the TLC plate. HPTLC is a powerful analytical method equally suitable for qualitative and quantitative analytical tasks.^[28]

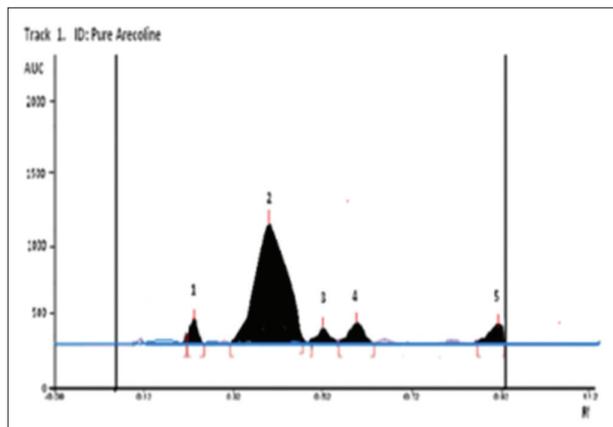


Figure 2: Chromatogram for pure areca nut

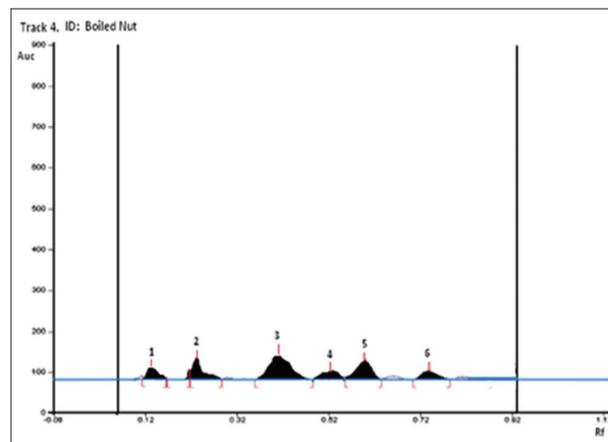


Figure 4: Chromatogram for boiled areca nut

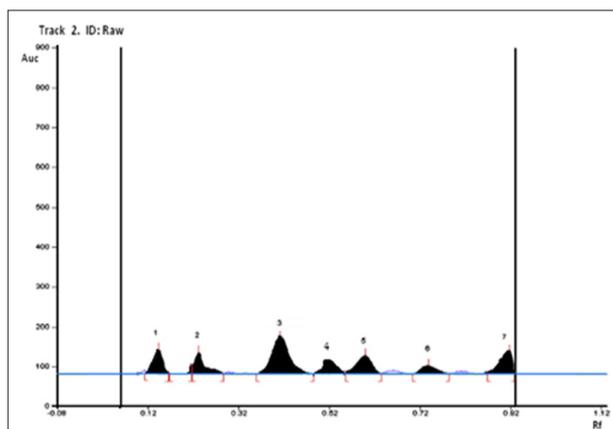


Figure 3: Chromatogram for raw areca nut

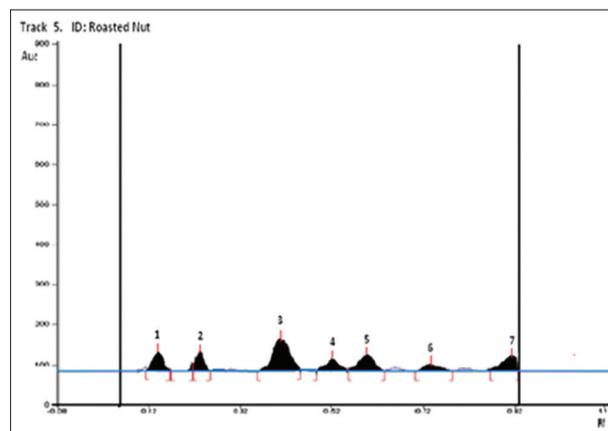


Figure 5: Chromatogram for roasted areca nut

HP TLC produces visible chromatogram complex, and information about the entire sample is available at a glance. Hence, multiple samples can be seen simultaneously and this helps straightforward comparison of the reference and the test samples. In addition to the visible chromatograms, analog peak data are also available from the chromatogram. They can be evaluated either by the image-based software Videoscan or more preferably by scanning densitometry with TLC Scanner, measuring the absorption and/or fluorescence of the substances on the plate [Figures 2-6].^[29]

To the best of our knowledge, there have been no reports on the assessment of arecoline in the various forms of areca nut preparations and its commercial derivatives in the Indian literature. Estimations of arecoline done around the globe show almost similar results to the ones we have obtained albeit using different methods [Table 6]. Increasing studies regularly and conclusively link arecoline with the causation of fibrosis in the connective tissue in OSF. Awareness of the high content of arecoline in the commercial and regular forms of consumption is vital data in education and prevention of the disorder. In

addition, establishment of arecoline as the primary fibrogenetic factor will encourage research into evaluation of blocking factors that may prevent the fibrosis.

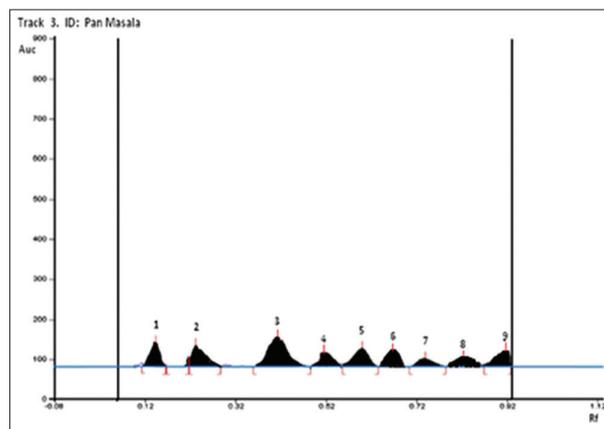


Figure 6: Chromatogram for pan masala

Table 6: Various studies that have assessed arecoline using chromatography

Author	Method	Study	Results
Awang, 1986 ^[10]	Gas-liquid chromatography	Arecoline content in 10 commercial samples from Mumbai was assessed	Sun-dried areca nut: 0-1.4% (mean 0.5%) Roasted areca nut: 0.4-1.3% (mean 0.9%) Boiled areca nut: 0.4-1.3% (mean 0.8%)
Aromdee et al. 2003 ^[13]	Reverse phase HPLC	Arecoline content in five different shapes of areca nut was determined. Samples - Round nuts: Small, medium, and large. Oblong: Medium and large	Small round nuts: 0.09% Medium round nuts: 0.04% Large round nuts: 0.02% Medium oblong: 0.12% Large oblong: 0.09%
Holdsworth et al. 1998 ^[15]	GC-mass spectral analysis	Green immature areca nuts	Identified presence of arecoline, guvacoline, nicotine, and other related alkaloids
Jantarat et al. 2013 ^[14]	HPLC	Comparison of arecoline content in ripe and unripe areca nuts	Unripe nut - 0.1434±0.0002; ripe nuts - 0.0944±0.0002% w/w
Cox et al. 2004 ^[12]	Ion-pairing reversed phase HPLC	Development of a new method to determine arecoline content in human saliva	-
Suzuki et al. 1995 ^[17]	Reverse-phase ion-pair HPLC	12 commercial areca nut samples	0.09-0.68% (mean 0.31%)
Yuan et al. 2012 ^[16]	HPLC	Determined arecoline content in 12 areca nut samples from Chinese mainland and evaluated effects on sperm motility	0.421%
Suleman and Wright 2013 ^[28]	GC and GC-MS	Evaluated arecoline content in four varieties of areca nut in Guam and Hawaii	Young Yapese- Mature Ugam Southern Guam Mature Ugam Central Guam Mature Changnga Central Guam
Pichini et al. 2003 ^[18]	HPLC with mass spectrometric detection	Evaluated arecoline concentration in newborn meconium, urine, and cord serum	0.006-0.008 µg/g in meconium
Kadi et al. 2013 ^[19]	Liquid chromatography/ion-trap mass spectrometry	Detected presence of arecoline in saliva of betel chewers	-

HPLC: High-performance liquid chromatography, GC: Gas chromatography, MS: Mass chromatography

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