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A Simple and Rapid HPLC Technique for Determination of Arecoline in Areca Nut (*Areca catechu* L.) Extract

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Abstract

A simple and rapid high-performance liquid chromatographic (HPLC) method for determination of arecoline extract from areca nut is reported. Arecoline is a major component cholinomimetic alkaloid found in areca nuts. It was extracted from dried seed powder to obtain high purity before analysis. The contents of arecoline in unripe and ripe areca nuts were compared. The analytical conditions for reverse-phase HPLC with UV detection were as follows: column, a 250 × 4.6 mm I.D., particle size 5 µm, Inertsil® ODS-3 (GL sciences Inc., Tokyo, Japan); mobile phase, a 88:12 (%v/v) mixture of acetonitrile: phosphate buffer (pH 5.9); column temperature, 25 °C; flow rate, 1 mL/min; and detection at 254 nm. The retention time of arecoline was 5.0 min. The calibration curve of the method was linear ($r^2 > 0.99$) in the 10 - 200 µg/mL range. Method inter-day precision (RSD) was 0.42 - 1.15 %, and % recovery was 103.23 ± 5.76 % indicating high precision and accuracy of the method. The contents of arecoline in unripe and ripe areca nuts were 0.1434 ± 0.0016 and 0.0944 ± 0.0002 %w/w of dried seed powder, respectively. The method was simple, rapid, precise, accurate and selective to determine arecoline in areca nut extract.

Keywords: Arecoline, areca catechu, HPLC method, determination

Introduction

Arecoline (methyl 1-methyl-1,2,5,6-tetrahydronicotinate) (**Figure 1**) is a cholinomimetic alkaloid found as a major component in areca nuts. It is well known that arecoline acts on both nicotinic and muscarinic receptors causing the stimulation of both sympathetic and parasympathetic nervous systems. People in southern Asian countries chew the areca seed alone or along with the betel leaf (*Piper betle* L.) to obtain sense of well being, a stimulating effect. Arecoline may have the potential for development as a drug for the treatment of Schizophrenia and Alzheimer diseases [1,2]. In herbal medicine, the areca nut has been used to treat parasitic worms since it contains tannins. The areca nut extract also shows potential pharmaceutical activities such as analgesic, anti-inflammatory and antioxidant [3]. Unfortunately, arecoline has also shown genotoxic, mutagenic and carcinogenic effects [4-6]. It is therefore important to be able to determine the content of arecoline in areca nut extract before applying further in pharmaceutics or cosmetics.

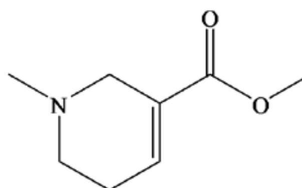

Figure 1 Structure of arecoline.

Arecoline was obtained from areca nut by extraction, but the content of arecoline in the areca

nut was low and the extraction efficiency of the traditional extraction method was also low. Previously, few quantitation methods have been reported for determination of arecoline content, these include ultraviolet (UV) spectrophotometry, gas chromatography (GC) and high performance liquid chromatography (HPLC). The HPLC technique is usually considered to be easier than GC and more sensitive and selective than UV spectrophotometry. A reverse-phase HPLC method for the determination of arecoline was developed by Aromdee *et al*. [7] to determine the contents of arecoline in different shapes of areca nut. Cox *et al*. [8] also successfully employed HPLC for the determination of arecoline in human saliva. From previous studies, the mobile phases used were prepared with the addition of an aqueous phase modifier and arecoline was eluted within 3 min [7]. For determination of active ingredients in vehicles (e.g. extract or preparation) or in biological fluids especially in plasma with HPLC using UV detection, the active ingredients would be extracted to obtain sufficient purity before analysis. In addition, the retention time would be long enough to avoid impurities of more polar compounds which normally are less retained in the reverse phase system.

The objective of this study was to develop and validate a HPLC method which is simple and rapid for determination of arecoline content in areca nut extract obtained from areca nuts harvested in Nakhon Si Thammarat, Thailand. The extraction method was optimized to obtain pure arecoline before analysis to separate any interference in order to maximize the specificity and sensitivity of the method. The contents of arecoline in unripe and ripe areca nuts were compared.

Materials and methods

Instrumentation

A Shimadzu HPLC system was used for the detection of arecoline. It consisted of an LC-10AT pump, DGU-14A degasser, SCL-10A controller, CTO-10AS column oven, SPD-10AV UV detector and a manual sample injector up to 20 μl was used (Shimadzu, Japan). The employed column was a 250 × 4.6 mm I.D., particle size 5 μm, Inertsil® ODS-3 (C-18) column (GL sciences Inc., Tokyo, Japan).

The mobile phase was varied and selected due to separation properties. It was composed of acetonitrile and 10 mM sodium phosphate buffer (pH 5.9) in the ratio of 88:12 (%v/v) at a flow rate of 1.0 mL/min. The UV detector was set up at 254 nm and the column oven was set at 25 °C.

Reagents and solvents

Arecoline hydrobromide (methyl 1-methyl-1,2,5,6-tetrahydronicotinate hydrobromide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). *Areca catechu* L. nuts were purchased locally from Nakhon Si Thammarat, Thailand. The green, immature areca nuts about 3 months old were collected and used as unripe areca nuts in this study. The yellow nuts (age ~ 8 months) were selected to use as ripe areca nuts. All solvents used were of either analytical reagent grade or HPLC grade.

The stock solution of standard arecoline was prepared by dissolving an accurately weighed amount of standard arecoline in methanol, and diluting with methanol to obtain the required working solution. The standard arecoline solution was prepared freshly before being used.

Validation study

The developed HPLC method was validated according to International Conference on Harmonisation (ICH) guideline [9] in terms of specificity, linearity, precision and accuracy, limit of detection (LOD) and limit of quantitation (LOQ). For the specificity study, it was carried out to check the absence of interference by the solvent and the degradation products of arecoline. Standard arecoline solutions (100 μg/mL) in methanol were submitted to accelerated degradation by leaving at ambient conditions for 24 h. A freshly prepared standard arecoline solution in methanol and arecoline solution with degradation products were analyzed in comparison with methanol in the HPLC conditions.

For the linearity study, five levels of concentration within the range 10 - 200 μg/mL arecoline were prepared. Each of the levels of concentration was prepared in triplicate, individually weighing the amount of standard arecoline to obtain three independent calibration equations. The linearity was evaluated by linear regression analysis, which was calculated by the

least square regression method with a non-weighting factor.

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). 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 (3 days). Three sample solutions of each three concentrations (40, 100 and 140 μg/mL) were prepared independently and assayed. For the accuracy study, it was determined by recovery of known amounts of standard arecoline. An accurately weighed amount of standard arecoline was dissolved and diluted to obtain the concentration of 40, 100 and 140 μg/mL in methanol before being assayed. It was carried out in triplicate.

For limit of detection (LOD) and limit of quantitation (LOQ), they were determined on the basis of response and slope of the regression equations as follows:

$$\mathrm{LOD} = \frac{3.3\sigma}{\mathrm{S}} \quad (1)$$

$$\mathrm{LOQ} = \frac{10\sigma}{\mathrm{S}} \quad (2)$$

where σ is the standard deviation of the response
S is the slope of the calibration curve

Arecoline extraction method and sample preparation

Fresh seeds were taken from areca nuts and dried at 60 °C for 72 h to obtain a stable weight. They were chopped and ground by roller mill and then passed through a 14-mesh sieve before extracted. Arecoline was extracted from dried seed powder using a Soxhlet extractor with dichloromethane. The method was modified from the method described by Holdsworth *et al*. [10] Briefly, five hundred grams of dried seed powder was draped in a slim white fabric and put in a Soxhlet extractor and extracted with 2.5 L dichloromethane which was made slightly basic (pH ~ 8) with ammonium hydroxide. The extraction was performed at 45 °C for 6 h. The cooled organic solvent (500 mL) was extracted with sulfuric acid solution (2 M, 3 × 25 mL). The aqueous extracts were collected and neutralized with aqueous sodium hydroxide (6 M), and then extracted with dichloromethane (3 × 10 mL). The organic extracts were dried over magnesium sulfate and evaporated using a rotary evaporator at 40 °C under reduced pressure to yield a dried brown color extract (1.16 % of dried seed powder). The extract was identified by thin layer chromatography (TLC) and Fourier transform infrared (FTIR) spectroscopy in comparison with standard arecoline. The TLC was carried out in 11 varieties ratio of ethanol and dichloromethane which were used as mobile phase of the systems. The FTIR spectra were collected using a Perkin-Elmer Spectrum-One FTIR spectrometer. The KBr disc method was used for solid samples. The extracts were kept in a desiccator and protected from light.

For sample preparation subjected to the HPLC method, the dried extracts were accurately weighed and dissolved in methanol, and then diluted with methanol to obtain the required concentration. All of the standards and extracts were filtered through a 0.45 μm syringe membrane filter (Type Millipore) and analyzed by HPLC. In comparison of the quantitation of arecoline in unripe and ripe areca nuts, the extraction procedure and the sample preparation were carried out in the same manner between unripe and ripe areca nuts. Each sample was prepared in triplicate for analysis.

Results and discussion

HPLC method development and validation

There are few HPLC methods available for arecoline quantification today and these are summarized in **Table 1**. Previous reports set the detector near 215 nm. In this study, the optimal wavelength for arecoline detection was established using UV absorbance scans over the range of 200 - 400 nm. Although detection at 215 nm was more sensitive, detection at 254 nm was more selective and also showed high signal response and did not pose a problem in terms of co-elution with impurities or solvent used. It means that if arecoline is quantified at 254 nm, the selectivity of the method is reached high. It was considered that 254 nm is the optimal wavelength for determination of arecoline extract. The flow rate of mobile phase was mostly 1 mL/min and the retention time of arecoline was rather short that

was within 5 min. Generally, the retention time might be adjusted by adjusting the composition of mobile phase and the pH [7]. In this study, the mobile phase was composed of a phosphate buffer (pH 5.9) and acetonitrile in a ratio of 12:88, and the retention time of arecoline was 5.0 min. If the ratio of acetonitrile is increased, the retention time of arecoline decreased and the peak shape was sharper. If the pH of the mobile phase increased, the retention time of arecoline would be increased due to the presence of more un-ionized form. Therefore, the mobile phase containing a phosphate buffer (pH 5.9) and acetonitrile at the ratio of 12:88 which gave the retention time of arecoline at 5.0 min was considered to be adequate for the method to determine arecoline extract. Since the retention time for determination of arecoline is long enough (5 min), this developed method might be able to use further for the determination of arecoline in plasma and pharmaceutical formulation since most protein and pharmaceutical vehicle interferences are usually eluted in a reverse-phase chromatographic system within 3 - 4 min. The mobile phase used in this study was simple containing a phosphate buffer and acetonitrile without any modifiers, and easy to prepare.

For validation of the method, **Figure 2** shows typical chromatograms obtained from the analysis of a solvent (methanol), standard arecoline and a sample of arecoline extract using the proposed method. It is clear that no peak interferences are observed in methanol at the same retention time for arecoline. Arecoline was eluted forming a symmetrical peak, well separated from the solvent front. It implied a good specificity of the method. The retention time observed (5.0 min) allows a rapid determination of the substance, which is important for routine analysis. The standard arecoline solution after accelerated degradation did not show degradation product peaks, it might conclude that arecoline in methanol is quite stable for at least 24 h at room temperature (30 °C).

Table 1 HPLC conditions for arecoline quantification.

Name of column	Mobile phase		Detector (nm)	Flow rate (mL/min)	Arecoline elution time (min)	Reference
	Aqueous phase modifier	Organic phase				
Nova-Pak C18 (150 × 3.9 mm)	Phosphoric acid 0.3 % and triethylamine 0.8 %	Water-potassium dihydrogen phosphate-acetonitrile (99:1:9, pH 5.5)	216	1	3.2	[7]
Luna C18 (250 × 4.6 mm, 5 μm)	Triethylamine 0.01 %	0.01M sodium hydrogenphosphate-acetonitrile (50:50)	215	1.2	3.1	[8]
Inersil ODS-3 C18 (250 × 4.6 nm, 5 μm)	None	0.01M sodium phosphate buffer (pH 5.9)-acetonitrile (12:88)	254	1	5.0	This study

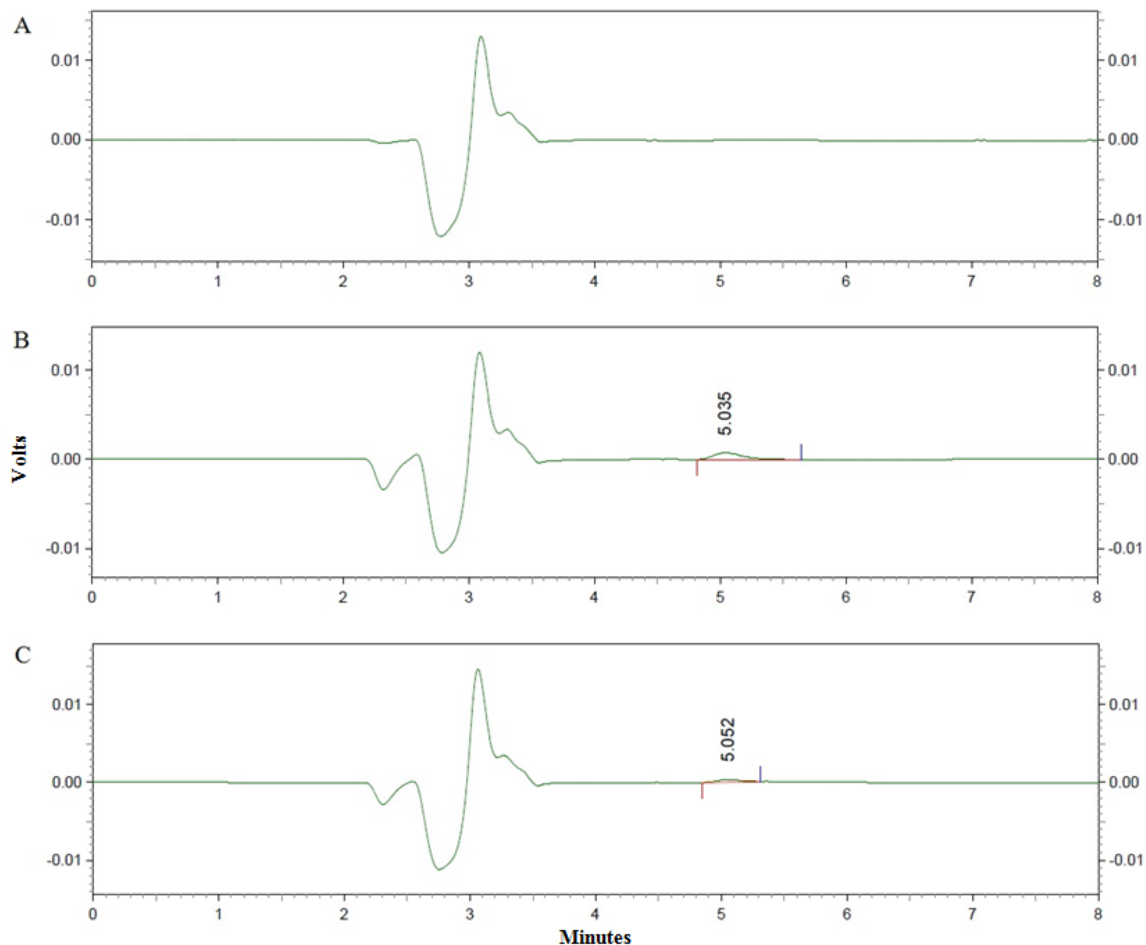

Figure 2 Typical HPLC chromatograms of (A) the solvent (methanol), (B) standard arecoline (100 µg/mL), and (C) arecoline extract.

The calibration curves for arecoline were constructed by plotting concentration versus peak area and showed good linearity in the 10 - 200 µg/mL range. A representative calibration curve of the average calibration curve data obtained from three independent calibration curves is shown in **Figure 3**. The representative linear equation was $y = 130.25x + 515.33$, with a correlation coefficient ($r^2 = 0.9961$) highly significant for the method (**Table 2**). The LOD and LOQ were found to be 2.86 and 8.69 µg/mL, respectively, indicating a high sensitivity of the method.

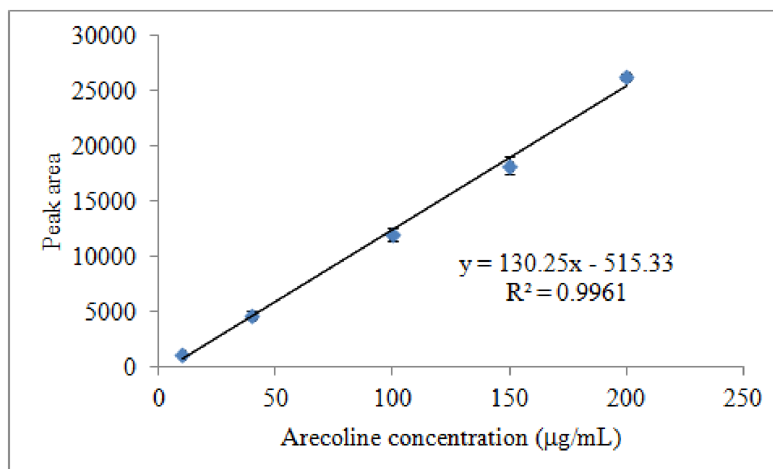

Figure 3 The calibration curve of arecoline (mean ± SD, n = 3).

Table 2 Results of regression analysis of data for the quantitation of arecoline by the proposed method.

Statistical parameters	Result
Regression equation	y = 130.25x + 515.33
Correlation coefficient (r^2)	0.9961
Standard error of slope	6.91
Standard error of intercept	398.28
Concentration range (µg/mL)	10 - 200 µg/mL

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as %RSD of a series of measurement. The experimental values obtained for the determination of arecoline solution are presented in **Table 3**. The %RSD of both intra-day and inter-day precision are lower than 2, indicating good precision. The accuracy of the method was determined and the mean recovery was found to be 103.23 ± 5.76 % (**Table 3**) indicating an agreement between the true value and the value found.

Table 3 Results of the determination of arecoline by the proposed method.

Arecoline concentration (µg/mL)	Experimental concentration (µg/mL)	%recovery	%RSD intra-day	%RSD inter-day
40	44.27 ± 0.27[a]	110.69 ± 0.69	0.63	
	42.08 ± 0.79[b]	105.21 ± 1.98	1.88	1.15
	44.16 ± 0.42[c]	110.40 ± 1.05	0.95	
100	96.85 ± 1.53[a]	96.85 ± 1.53	1.58	
	95.72 ± 1.22[b]	95.72 ± 1.22	1.27	1.10
	98.78 ± 0.43[c]	98.78 ± 0.43	0.43	
140	142.10 ± 1.37[a]	101.50 ± 0.98	0.96	
	141.59 ± 0.22[b]	101.13 ± 0.16	0.15	0.42
	152.25 ± 0.24[c]	108.75 ± 0.17	0.16	

[a]first day; [b]second day and [c]third day

Arecoline contents in unripe and ripe areca nuts

The extraction method for arecoline from areca nuts was specific for arecoline, resulting in obtaining less impurity as shown in the chromatogram (**Figure 2**) and TLC which showed only one spot of arecoline in 11 varieties of mobile phase systems as shown in **Figure 4**. The obtained arecoline extract was also confirmed by using FTIR. A similar pattern of spectra between arecoline extract and standard arecoline was observed as shown in **Figure 5**. The major absorption peaks at 1716 cm^{-1}, 1658 cm^{-1}, and 1106 cm^{-1} could be due to C=O, C=C, and C-N stretching vibrations, respectively. A peak at 1275 cm^{-1} could be due to the C-O stretching vibration. In addition, peaks at 1434 cm^{-1} and 1454 cm^{-1} corresponded to $–CH_3$ and $–CH_2–$ vibrations, respectively. These proved the success of arecoline extraction. In the first step of the extraction, an organic solvent was adjusted to alkaline (pH ~ 8) to drive arecoline to neutral form, since the pKa of arecoline is 6.8 [8]. When the pH is lower than the pKa, arecoline is in an ionized-form and could be extracted into the aqueous phase. The impurities were then removed by organic solvent. The pH was adjusted to alkaline again and arecoline was extracted into the organic phase. It would therefore obtain high purity arecoline.

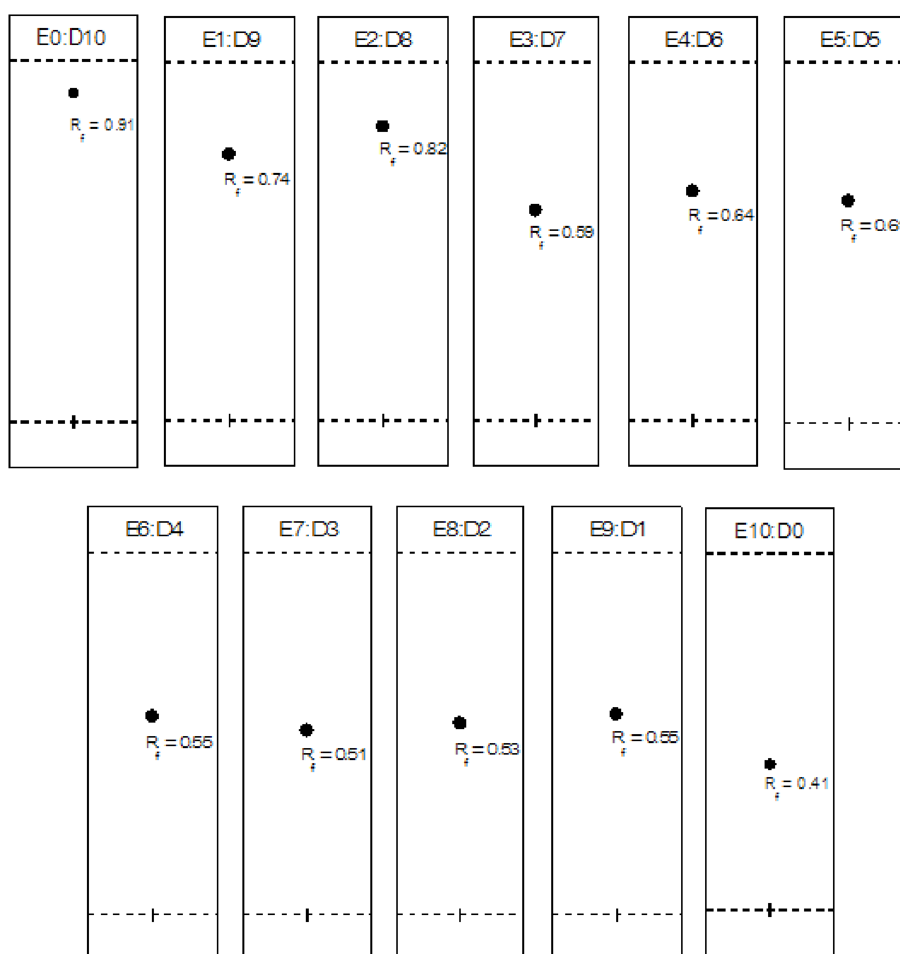

Figure 4 TLC showing only one spot of arecoline in 11 varieties of mobile phase systems. R_f is the retention factor; D is CH_2Cl_2; E is CH_3CH_2OH.

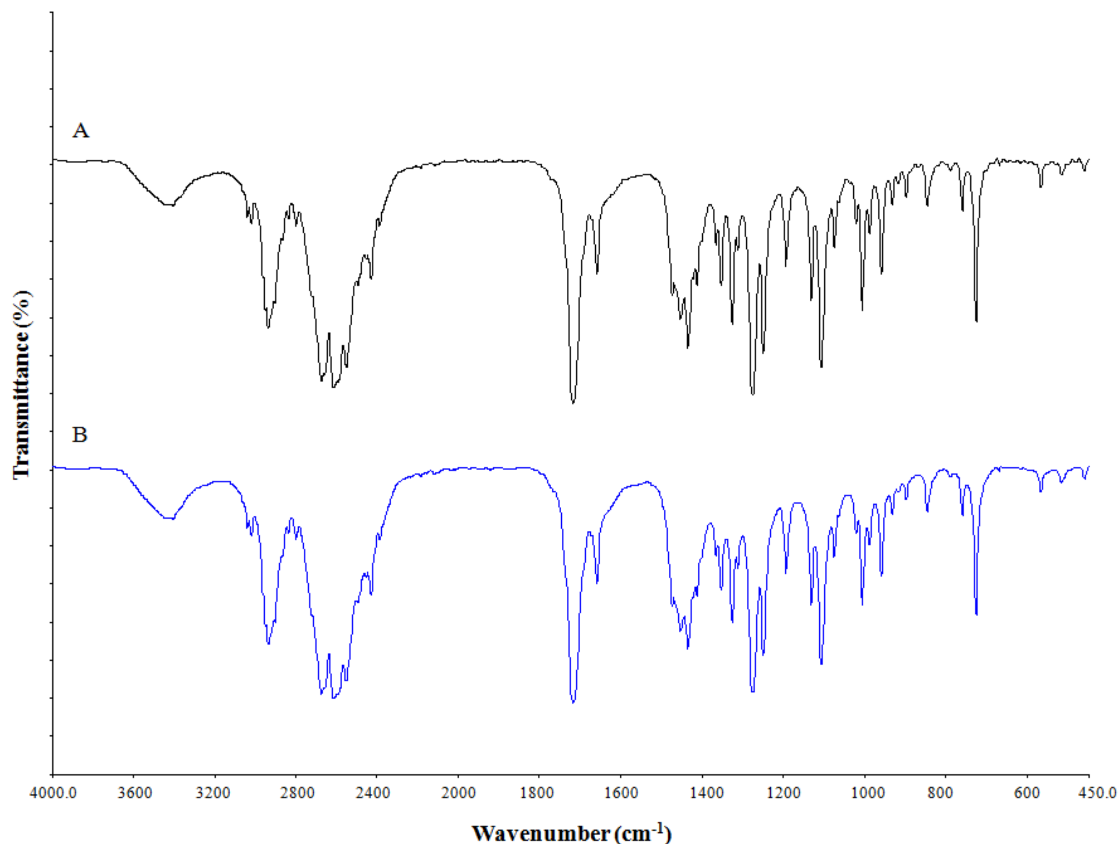

Figure 5 FTIR spectra of (A) standard arecoline and (B) arecoline extract.

The contents of arecoline in unripe and ripe areca nuts are calculated in %w/w of dried seed powder and are shown in **Figure 6**. In an equal amount of dried seed powder of unripe and ripe areca nuts, after extraction in the same manner in parallel, the contents of arecoline in unripe and ripe areca nuts were 0.1434 ± 0.0016 and 0.0944 ± 0.0002 %w/w, respectively. The contents of arecoline in areca nuts obtained from Northeastern Thailand were in the 0.02 - 0.12 % range [7] whereas the contents of arecoline in areca nuts grown in China were in the 0.22 - 0.56 % range [11]. Areca nuts grown in different areas (temperate area *vs* tropical area) might contain different amounts of arecoline. From the results of this study, we conclude that dried seed powder of unripe areca nuts contain significantly more arecoline than the dried seed powder of ripe areca nut ($p < 0.05$).

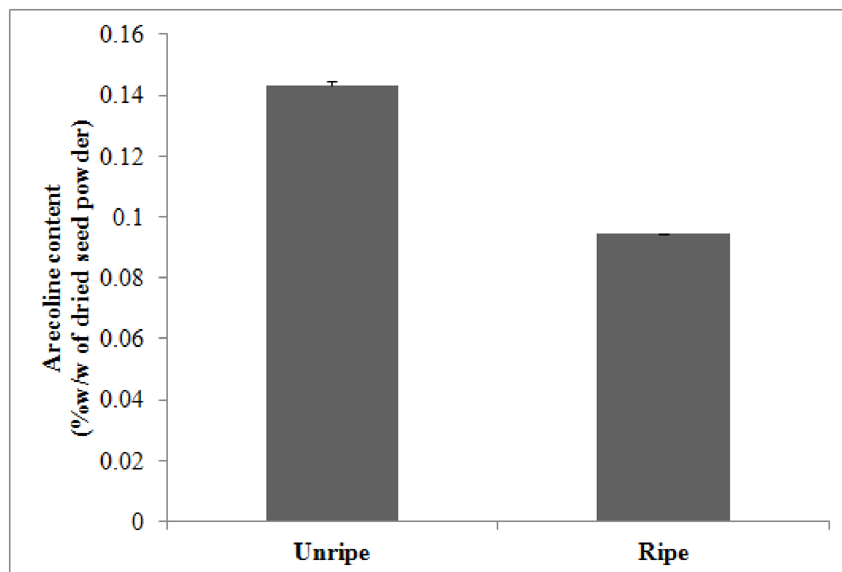

Figure 6 The contents of arecoline in unripe and ripe areca nuts (%w/w of dried seed powder) (mean ± SD, n = 3).

Conclusions

This study contributes to the establishment of an easy and rapid HPLC method for the determination of arecoline in arecoline extract from areca nut and for routine purpose analysis. The HPLC method was found to be simple, rapid, precise, accurate and selective. In addition, the specific extraction procedure for arecoline described in this study assists the ease in HPLC analysis. In summary, the proposed method can be used for arecoline analysis extracted from unripe and ripe areca nuts. For future work, the determination of arecoline content in pharmaceutical formulation and plasma will be studied.

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