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A new matrix for analyzing low molecular mass compounds and its application for determination of carcinogenic areca alkaloids by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Chia-Hsien Feng[a], Chi-Yu Lu[b,c,*]

[a] Department of Fragrance and Cosmetic Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan
[b] Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan
[c] Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan

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ABSTRACT

Arecoline is the main alkaloid present in the areca nut (or betel nut) and it has central nervous system effects. Its pharmacological activities induce the constriction of the bronchial smooth muscles, and stimulation of the lacrimal and intestinal glands. Chewing areca nut is harmful to health because this habit may increase the risk of the development of oral cancer. In this study, a fast method was provided for the determination of areca alkaloids by matrix-assisted laser desorption ionization (MALDI) mass spectrometer with a time-of-flight (TOF) analyzer. Traditionally the MALDI-TOF method was not suitable for the analysis of small molecular weight ($m/z < 600$) compounds because of the high background of the matrix. In this study, a new matrix was utilized to decrease the background interference effectively. After simple sample preparation, 1 μL sample supernatant was mixed with 1 μL matrix and then deposited on the target plate. This new matrix was also used to test the MALDI imaging experiment. Application of this MALDI-TOF method for trace analysis of arecoline by this new matrix in human plasma at sub μM level proved workable.

1. Introduction

Arecoline is the main alkaloid present in the areca nut (or betel nut) and it has several pharmacological effects. In many Asian cultures (such as India, Taiwan, and Southeast Asia), the areca nut is chewed along with betel leaf to obtain a stimulating effect. In herbal medicine, the areca nut has been used medicinally as a drug against parasitic worms [1]. A causal association between chewing betel and oral cancer has been well documented. Many studies have shown an association between areca nut chewing and the development of oral cancer leukoplakia and oral submucous fibrosis [2–7]. There are four main areca alkaloids: arecoline, arecaidine, guvacine and guvacoline [8]. Arecoline is the primary active ingredient responsible for central nervous system effects; it is known to have sympathetic and parasympathetic effects [9–14]. Arecoline also has genotoxic, mutagenic and carcinogenic potential [15–18].

Matrix-assisted laser desorption ionization (MALDI) is an ionization source used in mass spectrometry, allowing the analysis of large biomolecules and organic molecules. The ionization of the desired analytes is triggered by laser beam and the matrix is used to facilitate vaporization and ionization. Many kinds of matrices are commonly used for MALDI and these are the derivatives of benzoic acid, cinnamic acid and picolinic acid [19–24]. Among several useful matrices, α-cyano-4-hydroxycinnamic acid (CHCA) appears to be the most popular [25]. Because of the high concentration of matrices (commonly 10 mg mL^{-1}), the major source of background interference is from the matrices. For large molecule analysis, matrix interference does not generally happen at the mass region m/z above 600. This background interference may be derived from matrix clusters and large matrix clusters are often visible for CHCA [26]. The MALDI mass spectra are contaminated by the interfering peaks originating from traces of alkali metals, even when sample preparation is carefully performed [27–31].

In this study, we screened five chemical compounds that might be used as a MALDI matrix. These compounds contain the coumarin moiety and they might absorb the laser energy. We discovered a compound, 7-mercapto-4-methylcoumarin, which was a suitable matrix for analysis of small molecular compounds. The structure of the matrix is not acquired from derivatives of benzoic acid, cinnamic acid or picolinic acid. Compared with CHCA, the background interference of this matrix is decreased enormously.

* Corresponding author at: Department of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan. Tel.: +886 7 3121101 2370; fax: +886 7 313 3434.
E-mail address: cylu@kmu.edu.tw (C.-Y. Lu).

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We applied this matrix to analyze arecoline alkaloids and the results were acceptable. Furthermore, we used this matrix to test the MALDI imaging experiment. The signals of arecoline and arecaidine were collected and then distribution of arecoline and arecaidine in areca nut was displayed. Moreover, we applied this new matrix for quantitation of arecoline in human plasma by MALDI-TOF (time-of-flight) using a simple liquid–liquid extraction method. Application of this new matrix for detection of compounds of small molecular weight by MALDI-FOF proved feasible.

2. Experimental

2.1. Materials

Arecoline hydrobromide, arecaidine hydrochloride, guvacine, nicotine, nicotinamide, nicotinic acid, n-hydroxymethyl nicotinamide, nornicotine, cotinine, titanium dioxide, cetyltrimethylammonium bromide (CTAB), trifluoroacetic acid (TFA), 7-mercapto-4-methylcoumarin (compound 1), chromone-2-carboxylic acid (compound 2), chromone-3-carboxylic acid (compound 3), 6-fluorochromone-2-carboxylic acid (compound 4), 7-(diethylamino) coumarin-3-carboxylic acid (compound 5), α-cyano-4-hydroxycinnamic acid (CHCA) and acridine (internal standard, IS) were purchased from Sigma–Aldrich (St. Louis, MO). Ammonium bicarbonate, sodium hydroxide, potassium hydroxide, acetonitrile, methanol, n-hexane, ethyl acetate and toluene purchased from Merck (Darmstadt, Germany) were of chromatographic grade. Deionized water from a Milli-Q system, Millipore (Bedford, MA, USA), was used at all times. A stock solution of arecoline and arecaidine (1 mM) was prepared by dissolving these compounds in deionized water.

2.2. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis

A MALDI-TOF-MS system model Autoflex III Smartbeam equipped with a nitrogen laser radiating at 337 nm from Bruker Daltonics (Billerica, MA, USA) was used to obtain the data in positive ion reflector mode. All matrices (compounds 1–5) were dissolved at 10 mg mL^{-1} in acetonitrile containing 0.1% TFA. Each was vortex mixed for 30 s then centrifuged for 30 s before any aliquots were taken. One microliter of the sample was spotted on a stainless steel target (Bruker Daltonics) followed by addition of 1 μL matrix. All mass spectra were recorded by summing 2000 laser shots. The laser power was adjusted between 20% and 30% of its maximal intensity and the FlexAnalysis software was used for data processing (Bruker Daltonics).

2.3. Sample preparation for molar absorptivity test

Compounds 1–5 were dissolved in acetonitrile and the test concentration was 1 μg mL^{-1}. Compound solutions 1 mL were placed in the cuvette and then scanned by UV–visible spectrophotometer (Beckman Coulter, Fullerton, USA).

2.4. Sample preparation for MALDI imaging

Fresh areca nuts were purchased from a local street vendor. Sections (20 μm thick) were cut with a stainless steel blade and mounted onto conductive glass slides (Bruker Daltonics, Germany). Slide was not washed in ethanol because arecoline and arecaidine are easily soluble in ethanol. The matrix solution consisted of 200 mg matrix (compound 1) in acetonitrile containing 0.1% TFA. Fresh sections were carefully spray-coated using a TLC (thin layer chromatography) spraying device (Sigma–Aldrich, St. Louis, MO) made of glass. Repeated spray-drying cycles were performed at a 30 cm distance from the slide to obtain an optimal matrix deposition. Imaging experiments were performed using FlexImaging 2.0 software (Bruker) to control the analysis. The picture of the native section was used to teach the software. At each measuring point, 20 sufficiently intense laser shots were averaged per spectrum. The spot raster was set to 200 μm resulting in a good compromise between the resulting file size and spatial resolution power. Data analysis was also performed using FlexImaging 2.0 software.

2.5. Extraction of arecoline from human plasma

The sample preparation procedure in this study was very simple. IS (3 μg mL^{-1}) was prepared by dissolving acridine in toluene. Human plasma samples from healthy volunteers (no betel nut chewing history) were spiked with five different levels of arecoline solution to prepare the final arecoline concentrations in human plasma over the range of 0.2–10 μM for analytical calibration. Human plasma samples (10 μL) were placed in Eppendorf vials (500 μL) and then ammonium bicarbonate solution (200 mM, 20 μL) was added. The sample vials were vortexed (30 s) and centrifuged at 10,000 rpm (2 min). After centrifugation, 15 μL of the IS solution was added and sample vials were vortexed for 30 s then centrifuged at 10,000 rpm for 2 min. One microliter of the supernatant was spotted on a stainless steel target followed by addition of 1 μL matrix (compound 1). After co-crystallization of analytes and matrix, the target plate was subjected to MALDI-TOF.

3. Results and discussion

Arecoline and arecaidine are the main alkaloids present in the areca nut. Fig. 1 shows the structure of arecoline and arecaidine and the monoisotopic molecular weight of arecoline and arecaidine are m/z 155 and 141, respectively. High molecular weight analytes are suitable for MALDI-TOF analysis because of the background of the matrix. The molar ratio of matrix over analyte usually is over 5000 to 10,000. Therefore, the background interference is very serious because of the high concentration of the matrix, especially for the compounds of small molecular weight ($m/z < 600$). Accordingly MALDI-TOF analysis is suitable for the compounds of high molecular weight, such as peptides, proteins, polymers and dendrimers. If the background effect is diminished, MALDI-TOF is a powerful instrument for high throughput analysis of the compounds of small molecular weight. CHCA and its derivatives are the popular matrix and commonly used in MALDI-TOF. The CHCA matrix was not suitable for characterization of low molecular mass components because the matrix-related ions interfered with the component ions. The background of CHCA ranged from m/z 100 to 600. Therefore, analytes with $m/z \leq 600$ are not suitable to be detected by MALDI-TOF.

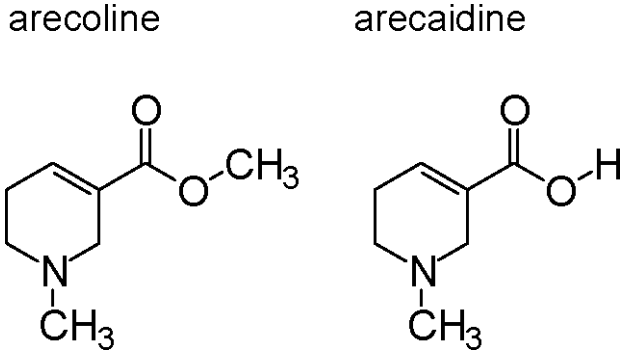


Fig. 1. The chemical structures of arecoline and arecaidine.

3.1. Matrix selection

Fig. 2A shows the background of CHCA matrix detected by MALDI-TOF. The background of the CHCA matrix is very high, and m/z 156.1 and 142.1 of CHCA matrix interfered with the $[M+H]^+$ of arecoline and arecaidine. The high background of CHCA might decrease the signal to noise ratio and then decrease the sensitivity of the instrument to detect the trace analytes. In order to screen a suitable matrix, five compounds containing coumarin ring were tested. Matrices used for MALDI-TOF need to contain two properties: absorb the laser energy near 337 nm and transfer a proton (or protons) from matrices to analytes to facilitate vaporization and ionization in positive ion mode. Many kinds of matrices have an aromatic ring to absorb the laser energy and a carboxylic acid to transfer a proton (or protons). Coumarin and its derivatives have the maximum absorbent wavelength near 337 nm. So we choose coumarin ring containing compounds as the candidate matrices for MALDI-TOF. We used a spectrophotometer to calculate the molar absorptivity of these five compounds. The results are shown in Table 1. In Table 1, compound 1 "7-mercapto-4-methylcoumarin" has the biggest molar absorptivity ($\log \varepsilon$) than other compounds at 337 nm. Furthermore, we used arecoline (10 μM) and arecaidine (10 μM) to test the relative intensity by using these five matrices to facilitate the vaporization and ionization of arecoline and arecaidine. The results are shown in Fig. 3. In Fig. 3, we could gain the high relative intensity of arecoline and arecaidine when using "7-mercapto-4-methylcoumarin" as the matrix. These results were equal to the phenomenon: the larger the molar absorptivity, the higher the intensity.

In this study, a compound "7-mercapto-4-methylcoumarin" was used as a new matrix for small mass compound analysis by MALDI-TOF. The structure of this compound was not a derivative of benzoic acid, cinnamic acid and picolinic acid. The λ_{max} of this compound is near 337 nm (λ_{max} = 322 nm in methanol) and it could transfer a proton after analyte ionization triggered by a laser beam. Fig. 2B shows the background of this new matrix detected by MALDI-TOF. Comparing Fig. 2B with Fig. 2A, the background of this new matrix is lower than the background of CHCA. Fig. 2C shows MALDI-TOF mass

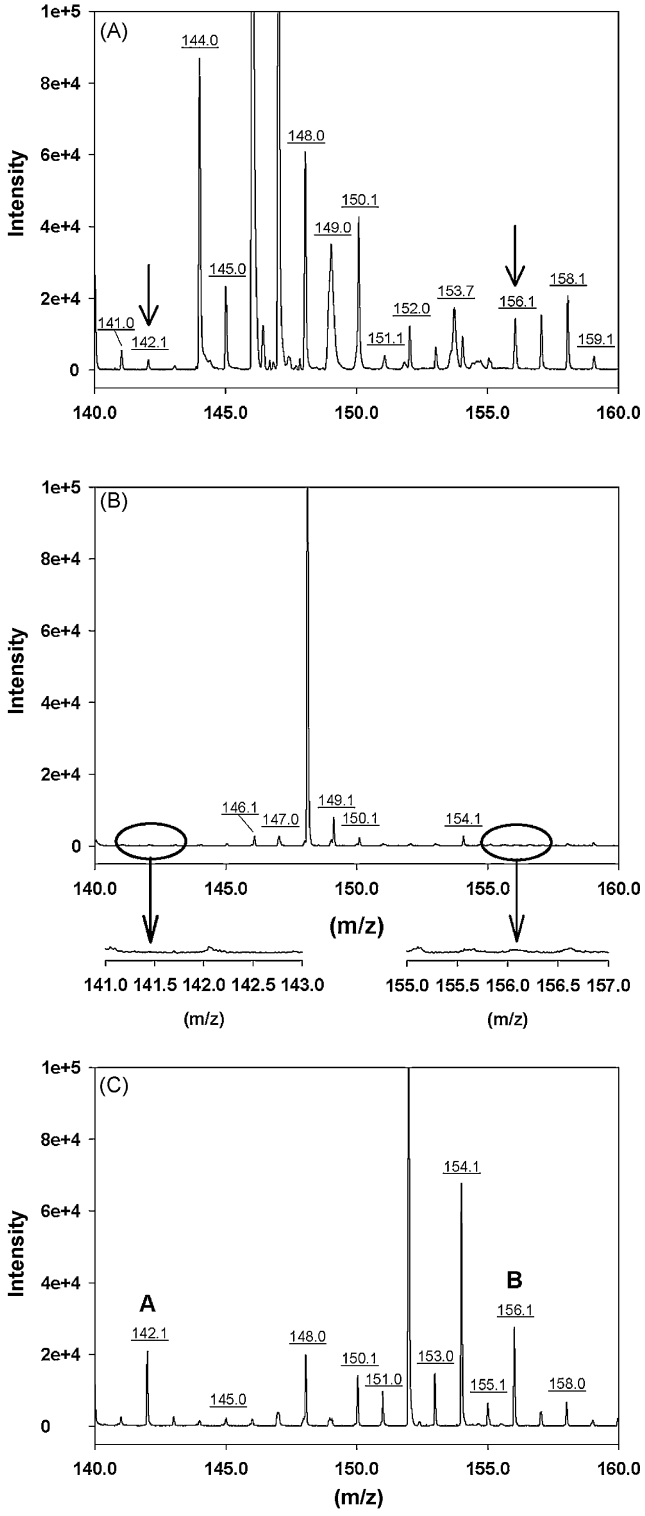
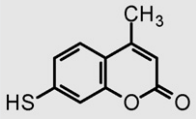
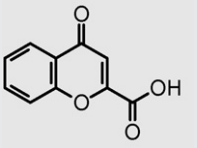
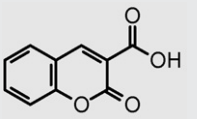
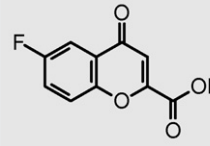
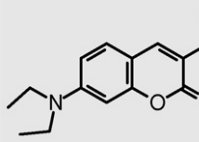


Fig. 2. The $[M+H]^+$ of arecoline and arecaidine are m/z 156.1 and 142.1. Mass spectra of MALDI-TOF: (A), the background of CHCA interfered with arecoline and arecaidine that were indicated by arrows; (B), the background of new matrix did not interfere with arecoline and arecaidine; (C), the $[M+H]^+$ of B (arecoline) and A (arecaidine) at 10 μM.

Table 1
Chemical structure and $\log \varepsilon$ of MALDI matrix.

Compound	Chemical structure	$\log \varepsilon$ in 337 nm
1		4.06
2		2.72
3		3.71
4		3.18
5		2.87

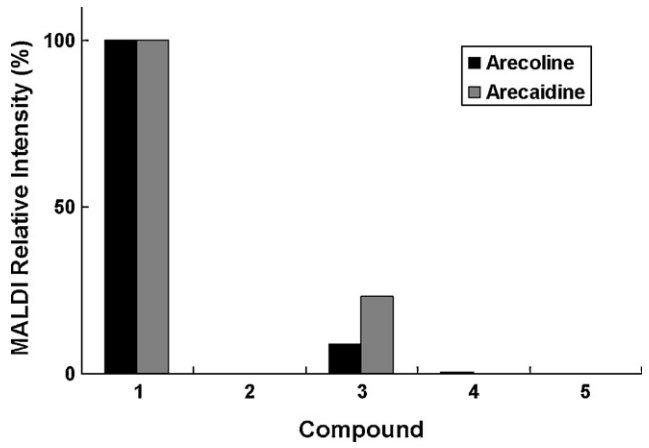


Fig. 3. The relative intensity of arecoline and arecaidine tested by different matrices.

spectra of 10 μM arecoline and arecaidine. We use this new matrix to test the metabolite of arecoline and some pyridine alkaloids that we can gain, including guvacine, nicotine, nicotinamide, nicotinic acid, n-hydroxymethyl nicotinamide, nornicotine and cotinine. We could also obtain the signals from these compounds at the concentration of 10 μM.

Arecoline and arecaidine could be ionized by this compound, no background interference could be found in the mass spectrum and we could see the $[M+H]^+$ monoisotope of arecoline and arecaidine at the m/z 156.1 and 142.1 (see Fig. 2B and C). Therefore, this compound is a suitable matrix for the detection of analytes of small molecular weight ($m/z < 600$) by MALDI-TOF. We also tested this matrix to assist in ionizing high molecular compounds ($m/z > 1000$, such as proteins, peptides, polymers and dendrimers) and detected by MALDI-TOF. Contrary to our expectations, we could not get any signal when using this coumarin compound as the matrix to detect these high molecular compounds (data not shown). This compound

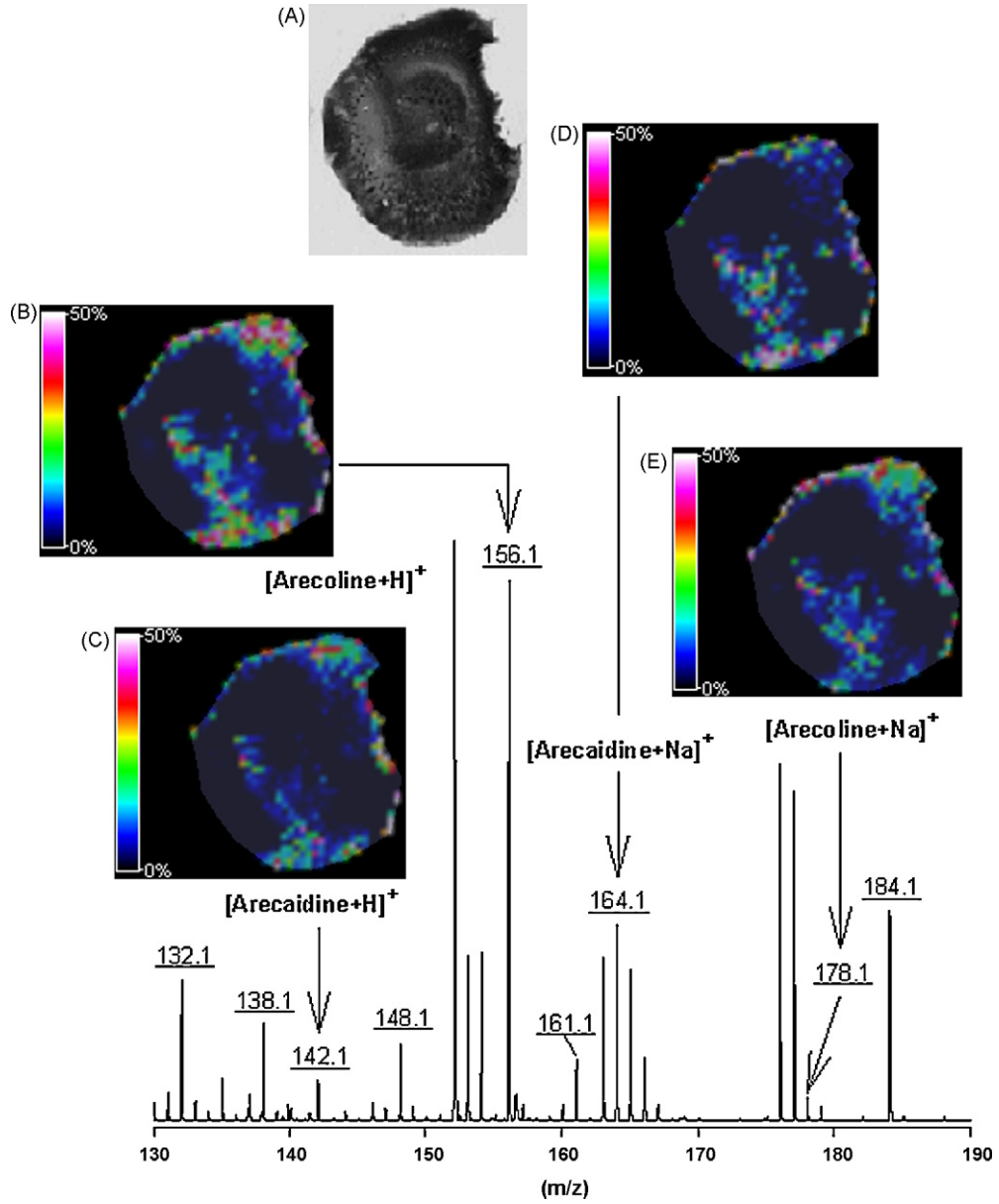


Fig. 4. The MALDI-TOF mass signals were transferred into images. The distribution of arecoline and arecaidine in fresh areca nut section was tested by MALDI imaging: (A), image of fresh nut tissue; (B), $[M+H]^+$ of arecoline; (C), $[M+H]^+$ of arecaidine; (D) $[M+H]^+$ of arecaidine and (E) $[M+H]^+$ of arecoline. The intensities of arecoline and arecaidine relating to each isobar are shown with specific colors. Rainbow colors represent the different intensities (black to white color ranged from 0% to 50%) of arecoline and arecaidine in the tissue.

"7-mercapto-4-methylcoumarin" might be a new matrix for selective analysis of small molecular weight compounds by MALDI-TOF.

We also selected three MALDI approaches for low molecular weight analytes to detect arecoline and arecaidine. In the first method [32], CHCA was used as the matrix and cetyltrimethylammonium bromide (CTAB) was used as a matrix-ion suppressor. In the second method, CHCA and 9-aminoacridine were utilized as the binary matrices for background suppression [33]. In the third method, we used TiO_2 as a matrix for the MALDI analysis [34]. Compared our method with these methods, the relative intensity (%) of (arecoline, arecaidine) by using compound 1, binary matrices, CHCA with CTAB and TiO_2 as the matrix are (100, 100), (67, 10), (27, 37) and (3, 9). Compound 1 is indeed a suitable matrix for MALDI-TOF.

3.2. MALDI imaging

Moreover, we used "7-mercapto-4-methylcoumarin" to detect the signals of arecoline and arecaidine in the MALDI imaging experiment. MALDI imaging is a technique that shows the spatial distribution of desired analytes within thin slices of animal or plant tissues. In this study, fresh nut tissue was used for MALDI imaging tested. The results of MALDI imaging are shown in Fig. 4. In Fig. 4, the distribution of arecoline and arecaidine in fresh areca nut were detected by MALDI-TOF imaging and the signals were transferred into images. The expected signals for arecoline and arecaidine would occur at m/z 156.1 and 142.1 as the $[M+H]^+$ ion. The images generated from the product ions of the mass spectra show the different distribution of arecoline and arecaidine in the nut tissue. The intensities of arecoline and arecaidine relating to each isobar are shown with specific colors. Rainbow colors represent the different intensities (black to white color ranged from 0% to 50%) of arecoline and arecaidine in the tissue. Arecoline and arecaidine are the major components in the areca nut. According to the molecular weight, we could also see the expected $[M+Na]^+$ of arecoline and arecaidine in the fresh nut at m/z 178.1 and 164.1 (see Fig. 4D and E). Because the core of the areca nut is hollow, the signal of arecoline and arecaidine in this area is absent.

3.3. Optimization of the extraction procedure for arecoline

In order to test the sensitivity of MALDI-TOF by using the compound "7-mercapto-4-methylcoumarin" as the new matrix, different concentrations of arecoline spiked in human plasma were tested. We used the liquid–liquid extraction method to extract arecoline. Some parameters affecting the extraction (see Section 2.5) were studied including base, base concentration and extraction solvent. The concentration of arecoline used for the study was 10 μM. The effects of the parameters on the extraction of the arecoline were evaluated and optimized based on the peak area ratios of the arecoline (m/z 156.1) to the IS (m/z 180.1).

The effects of different bases (ammonium bicarbonate, potassium hydroxide and sodium hydroxide) on extraction efficiency of arecoline were studied. The relative extraction efficiency by adding ammonium bicarbonate, potassium hydroxide and sodium hydroxide are 100%, 50% and 26%, respectively. The results (Fig. 5A) indicate that ammonium bicarbonate is the best for arecoline extraction. The effects of the ammonium bicarbonate at varied concentrations (0–800 mM) on the extraction of arecoline were tested. The results (Fig. 5B) indicate that the extraction efficiency is better when the concentration of ammonium bicarbonate is 200 mM. The effects of water-insoluble organic solvents (toluene, ethyl acetate and n-hexane) on extraction of arecoline were elucidated. The relative extraction efficiency of toluene, ethyl acetate and n-hexane are 100%, 14% and 9%, respectively. The results (Fig. 5C) indicate that toluene is the solvent of choice for the extraction of arecoline from human plasma.

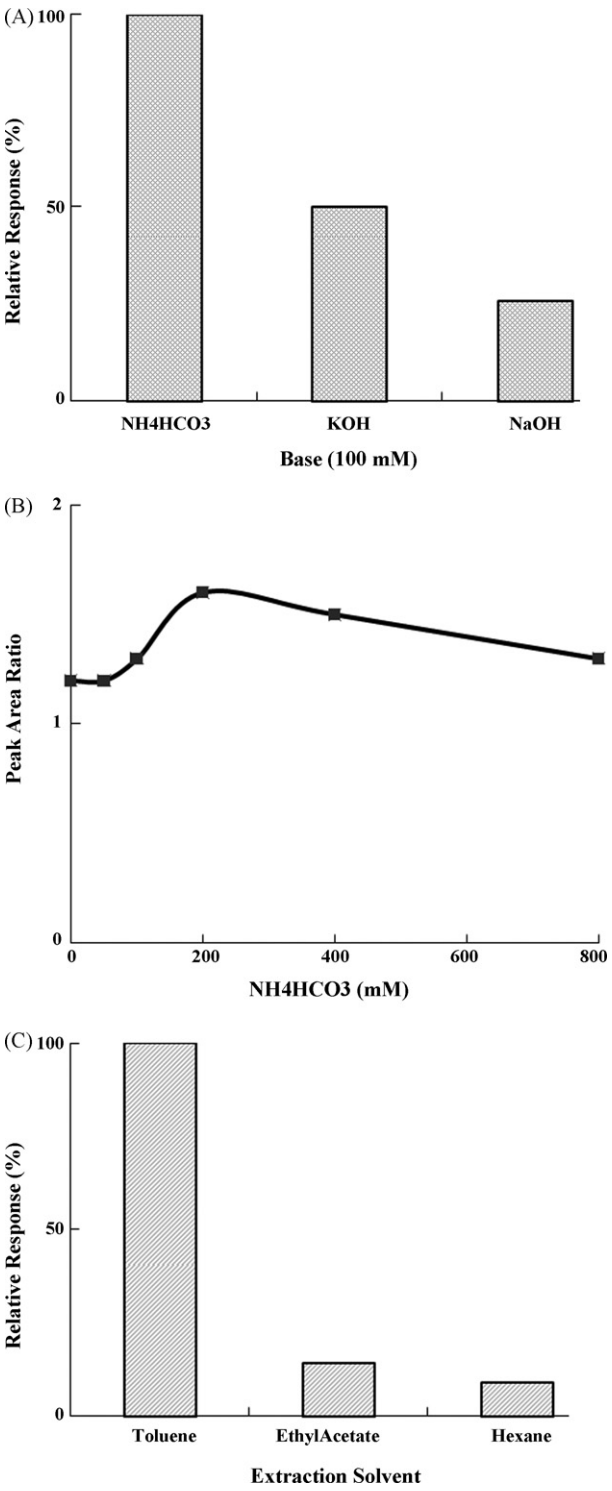


Fig. 5. Optimized conditions for extraction of arecoline; (A) effects of bases on the extraction of the arecoline (B); effects of ammonium bicarbonate concentration on the extraction of the arecoline and (C) effects of organic solvents on the extraction of the arecoline.

3.4. Analytical calibration, precision and accuracy

Quantitation of arecoline was performed by integrating the peak area of arecoline (m/z 156.1) and IS (m/z 180.1) for a series of con-

Table 2
Precision and accuracy for the determination of arecoline spiked in human plasma.

Concentration known (μM)	Concentration found (μM)	R.S.D. (%)	R.E.[c] (%)
Intra-day[a] ($n = 5$)			
0.500	0.460 ± 0.069	15.00	−8.00
2.000	2.108 ± 0.281	13.33	+5.40
8.000	7.798 ± 0.702	9.00	−2.53
Inter-day[b] ($n = 5$)			
0.500	0.432 ± 0.044	10.19	−13.60
2.000	1.960 ± 0.177	9.03	−2.00
8.000	8.152 ± 0.249	3.05	+1.90

[a] Intra-day assay variance from analysis of arecoline at five intervals on a single day.
[b] Inter-day assay variance from analysis of arecoline on five consecutive days.
[c] R.E. calculated from (value found-value known)/value known.

centrations. Isotope-labeled IS is very expensive and sometimes it is not easy to obtain. According to our experience, choose an IS close to the mass of the desired molecule is an alternative way for MALDI-TOF. In this study, we choose acridine as the IS because of the mass of acridine is close to the mass of arecoline. The calibration curve for the analysis of arecoline in spiked plasma at five different levels of arecoline over the range of 0.2–10 μM was constructed. The linearity was evaluated between the peak area ratio of arecoline to the IS as ordinate (y) and the concentration μM of the arecoline as abscissa (x). The linear equation for the concentration vs the ratio of peak area was $y = (0.1204 \pm 0.0134)x - (0.0066 \pm 0.0043)$ with a correlation coefficient of 0.995 ($n = 5$). The results showed that the peak area ratio was linearly related to the arecoline concentration for the range 0.2–10 μM and good linearity was attainable. The precision (relative standard deviations, R.S.D.) and accuracy (relative error, R.E.) of the method were studied based on the peak area ratios for the analysis of arecoline at three levels, 0.5, 2 and 8 μM. Table 2 indicates that the R.S.D. and R.E. values for the intra- and inter-day analyses of the spiked plasma are all below 15.1% and 13.7%, respectively. The recovery of this method ranged between 94% and 112%. The detection limit of arecoline was about 0.1 μM (S/N = 3). The sensitivity of this method is sufficient for monitoring arecoline in human plasma by using only 10 μL plasma. According to our study, trace analysis of arecoline by this new matrix could be attained at sub μM level by MALDI-TOF.

4. Conclusions

A new matrix "7-mercapto-4-methylcoumarin" was screened and utilized to analyze carcinogenic alkaloids by MALDI-TOF. This compound contains the coumarin moiety that could absorb laser energy and facilitate the vaporization and ionization of desired compound. This compound does not have a carboxylic group, but a proton also could transfer from the –SH group to the desired analytes. This compound, 7-mercapto-4-methylcoumarin, was used for the first time as the MALDI matrix. This matrix was suitable for analysis of small molecular weight compounds (m/z below 600) by MALDI-TOF. Application of this matrix to detect other compounds could be anticipated.

Acknowledgements

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