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Laurie Gheddar, Francois-Xavier Ricaut, Alice Ameline, Nicolas Brucato, Roxanne Tsang, et al.. Testing for Betel Nut Alkaloids in Hair of Papuans Abusers using UPLC–MS/MS and UPLC–Q-Tof-MS. *Journal of Analytical Toxicology*, 2020, 44 (1), pp.41-48. <10.1093/jat/bkz045>. <hal-02273344>

HAL Id: hal-02273344

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Submitted on 29 Sep 2023

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Testing for Betel Nut Alkaloids in Hair of Papuans Abusers using UPLC-MS/MS and UPLC-Q-Tof-MS

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Abstract

Betel nut is the fruit of Areca palm, growing in Papua New Guinea. Mixed with limestone and stick mustard, arecoline and guvacolone, which are present in betel nut, are hydrolyzed into arecaidine and guvacine, respectively. As part of the study on dietary habits of Papuans residents, our laboratory was asked to analyze the four alkaloids in hair to document long-term exposure. Hair samples were collected from 19 adult subjects (males = 11; females = 8), by some of the authors, and were sent to the laboratory for analysis. The four alkaloids have very similar chemical structures. In order to accurately identify the drugs, two methods were developed. First, the compounds were identified using an ultra-high-performance liquid chromatography system coupled to time-of-flight mass spectrometry. Then, they were quantified by an ultra-high-performance liquid chromatography system coupled to tandem mass spectrometry. After decontamination with dichloromethane, hair samples were cut into very small segments and 20 mg were incubated in methanol for 2 h 30 min in an ultrasound bath. After cooling, the methanol was evaporated to dryness in presence of 20- μ L octanol to prevent volatilization. Nicotine-d₄ was used as an internal standard. Linearity was observed for concentrations ranging from the limit of quantification to 20 ng/mg for arecoline, arecaidine, guvacine and guvacolone. Measured concentrations were in the range 60 pg/mg to 18 ng/mg for arecoline ($n = 19$), 14 pg/mg to 2.5 ng/mg for guvacolone ($n = 11$), 63 pg/mg to 3.8 ng/mg for arecaidine ($n = 11$) and 100 pg/mg to 3.2 ng/mg for guvacine ($n = 6$). There was no correlation between concentrations of arecoline and arecaidine (ratio from 0.01 to 0.18) and guvacolone and guvacine (ratio from 0.06 to 3.50). However, the identification of these substances in hair is a good marker of consumption of betel nut and allows us to document a local practice that remains difficult to evaluate just by questioning.

Introduction

In Papua New Guinea (PNG), betel nut or areca nut, also called 'buai' is the fruit of *Areca catechu* palm. This nut is highly consumed by the local population and is the object of intense commercial activity, including purchase, sale and product manufacturing within the island. The betel nut is mixed with limestone powder (calcium oxide), named 'kambang' and stick mustard, which is a kind of bean called 'daka'. Several types of betel nuts exist but the nut is

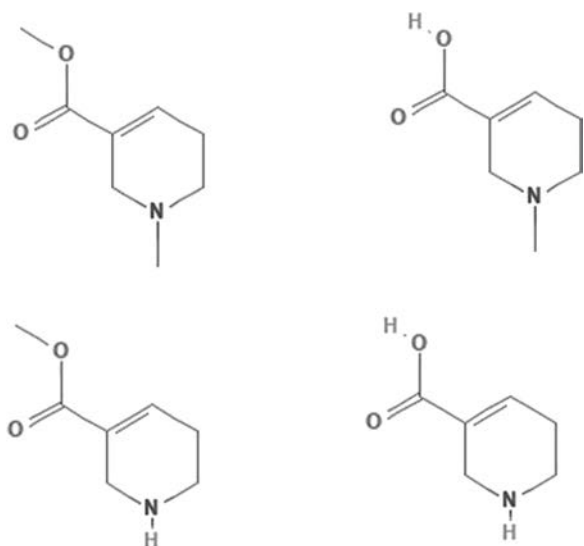


Figure 1. Top to bottom, left to right: arecoline (MW = 155.2 g/mol), arecaidine (MW = 141.2 g/mol), guvacoline (MW = 141.2 g/mol), guvacine (MW = 127.1 g/mol)

generally round or oval and can measure up to 5 cm long. The nut is initially green and has a sweet taste. At maturity, it becomes yellow until brownish and has a bitter taste. The lime powder can be produced with shells, mollusks and corals. These products are burned and crushed until a powder is obtained. The three ingredients are processed as follows: (i) the green betel nut is broken with teeth and chewed; (ii) the mustard stick is dipped in the lime powder; and (iii) the three ingredients are chewed together. This mixture gives a red color that colors the saliva as well as the teeth and is chewed all throughout the day and finally spat in the streets or in bins by the residents (1–3).

Betel nut is composed of four major alkaloids namely arecoline (up to 1% of dry weight), guvacoline, arecaidine and guvacine (Figure 1) (4–6). Moreover, in presence of lime (basic conditions), arecoline and guvacoline are hydrolyzed into arecaidine and guvacine, respectively (7–8).

The use of betel nut causes some positive effects such as euphoria, relaxation and improved concentration. These substances are central nervous system stimulants, muscarinic agonists with psychoactive, stimulating effects. Guvacine and arecaidine are strong inhibitors of Gamma-Aminobutyric Acid (GABA) uptake and contribute to psychoactive effects, too. They also cause negative effects such as salivation, vertigo, myosis, tremor, bradycardia and asthma attacks. It also described that betel nut chewing can cause anxiety, irritability and insomnia (4, 9–11).

Due to the stimulant effects, the regular use of betel nut in PNG is associated with psychological addiction. Abuse of betel nut is also the principal cause of mouth and esophagus cancer in PNG. Finally, it is a public health problem with the spread of tuberculosis (3) as it has been observed that this practice can enhance population diseases.

As part of the study on dietary habits and phenotypic and genetic diversity of the residents, our laboratory was asked to analyze the active substances in hair to document long-term exposure to betel nuts.

The simultaneous identification and quantification of four betel nut alkaloids in hair has not yet been published. In the literature,

arecoline has been determined in several matrices, such as saliva, plasma, urine, breast milk, meconium, hair and, of course, raw areca nut (12–18, 20–23). Arecaidine was determined in saliva, plasma, teeth, urine and areca nuts (12–13, 16–17, 19, 22–23). Guvacine was analyzed in teeth, urine, saliva and areca nuts (19, 22–23) and guvacoline in saliva, urine and areca nuts (21–23). Only Marche *et al.* (14) have identified arecoline in hair of 11 abusers, with concentrations ranging from 300 pg/mg to 1.70 ng/mg (14).

Chromatographic separation of these four alkaloids seems difficult. The drugs have very similar chemical structures (Figure 1) and molecular weights that are close or identical (for arecoline and guvacoline). In order to identify accurately the drugs, two methods were developed. First, the compounds were identified using an ultra-high-performance liquid chromatography system coupled to time-of-flight mass spectrometry (UPLC-q-ToF-MS). Then, they were quantified by an ultra-high-performance liquid chromatography system coupled to tandem mass spectrometry (UPLC-MS/MS) using the same chromatographic conditions.

Experimental

Hair specimens

Hair samples were collected from 19 adult subjects (males = 11; females = 8), by some of the authors, in PNG and were sent to the laboratory for analysis via courier, at ambient temperature. Hair specimens measured between 1 and 5 cm in length, and were black or brown in color. The orientation root to tip was not maintained. The 19 subjects confirm that they did not cosmetically treat their hair before sampling and that they were abusing betel nut for more than 6 months. As there was no attempt to correlate the habits of consumption with the measured concentrations, frequency and intensity of consumption were not recorded.

Each hair specimen was received in a plastic bag and stored at ambient temperature in the laboratory before analysis. Blank hair for method validation was obtained from laboratory staff.

Characteristics of the tested specimens are presented in Table I.

Table I. Characteristics of the Hair Samples

Sample	Gender	Color	Length (cm)	Age
1	F	Brown	2	20–30
2	F	Black	1	20–30
3	F	Black	3	20–30
4	M	Brown	1	20–30
5	F	Brown	1	20–30
6	F	Brown	4	>60
7	M	Black	2	50–60
8	F	Brown	4	40–50
9	M	Black	1	>60
10	F	Black	2	20–30
11	M	Brown	4	20–30
12	M	Black	2	20–30
13	M	Brown	4	20–30
14	M	Black	2	20–30
15	M	Black	1	20–30
16	F	Brown	5	20–30
17	M	Black-gray	3	20–30
18	M	Black	1	20–30
19	M	Black	5	20–30

Chemicals and reagents

Arecoline hydrobromide, guvacoline hydrobromide and nicotine d4 were obtained from Sigma Aldrich (Steinheim, Germany), from ApexBio Technology LLC (Houston, USA) and from Cerilliant (Saint Louis, MO, USA), respectively. Arecaidine hydrochloride and guvacine hydrochloride were obtained from Santa Cruz Biotechnology (Dallas, USA). Methanol for HPLC Isocratic Grade (MeOH) and dichloromethane were purchased from VWR Chemicals Prolabo (Fontenay-sous-Bois, France) and 1-octanol from Sigma Aldrich (Steinheim, Germany).

The standard solutions, prepared in MeOH, were stored at 4°C.

Sample treatment

Decontamination

The hair locks were decontaminated twice with 5 mL of dichloromethane for 1 min at room temperature and dried. Decontamination solutions were stored at 4°C and analyzed for a second time.

Preparation and extraction of hair

The hair was cut into very short segments (<1 mm) using scissors. Given the curly nature of all the specimens, there was no attempt to do segmentation. 20 mg of decontaminated hair were incubated in 1 mL MeOH for 2 1/2 h in an ultrasonic bath, in the presence of 20 ng of nicotine d4 used as internal standard. After centrifugation step, MeOH phase was collected and evaporated to dryness under a nitrogen flow, in presence of 20- μ L octanol to prevent volatilization (24). The residue was dissolved in 30 μ L of the MeOH and was injected twice: 1 μ L into the UPLC-q-ToF-MS system and 2 μ L into the UPLC-MS/MS system.

Instrumentation

Ultra-high-performance liquid chromatography system coupled to time-of-flight mass spectrometry

Chromatographic separations were performed with a liquid chromatography Waters Acquity UPLC™ system (Waters corporation, Milford, MA, USA) using an Acquity UPLC HSS C18 column (150 mm \times 2.1 mm, i.d. 1.8 μ m particle size). The column temperature was set to 50°C, and the flow rate at 0.3 mL/min. The mobile phase consisted of water, 0.1% formic acid (eluent A) and MeOH, 0.1% formic acid (eluent B). Total analysis time was 8.0 min and the following gradient pattern (eluent A) was used for the separation: 0 min, 98%; 4.00 min, 98%; 4.20 min, 5%; 4.60 min, 5%; 4.70 min, 98%; and 8.00 min, 98%. Arecoline, guvacoline, arecaidine, guvacine and nicotine-d4 were eluted in 1.27, 1.27, 1.10, 1.08 and 1.24 min, respectively.

Detection was performed using a high resolution (XEVO™ G2XS Q-TOF, Waters Corporation, Milford, MA, USA) mass spectrometer operating in positive-ion mode and in sensitivity mode. The desolvation gas flow was set to 600 L/h at a temperature of 400°C with the cone gas set to 20 L/h and the source temperature set to 150°C. The capillary voltage and the cone voltage were set to 2000 and 25 V, respectively. Leucine-enkephalin was used as the lock mass (m/z 556.2766) for accurate mass calibration and introduced using the LockSpray interface at 10 μ L/min and a concentration of 200 pg/ μ L in 50% aqueous ACN, 0.1% formic acid. In MS scanning, data were acquired from 50 to 1000 m/z . As for MS/MS fragmentation of target ions, collision energy ranging from 10 to 40 V was applied. Unifi software was used for data, chromatograms and spectra acquisition.

Ultra-high-performance liquid chromatography system coupled to tandem mass spectrometry

Chromatographic separations were performed with identical equipment than UPLC-q-ToF-MS. The applied parameters (column temperature, mobile phase, gradient pattern) were the same compared to UPLC-q-ToF-MS, except for the flow rate, which was 0.2 mL/min. Arecoline, guvacoline, arecaidine, guvacine and nicotine-d4 were eluted in 1.85, 1.85, 1.65, 1.65 and 1.85 min, respectively.

Detection was performed using a triple quadrupole (XEVO™ TQD, Waters Corporation, Milford, MA, USA) mass spectrometer equipped with a Z-spray™-electrospray ionization source and used in the positive mode. Nitrogen was used as drying gas as well as nebulizing gas. The desolvation gas flow was set to 600 L/h and the desolvation temperature was 400°C. The source temperature was 150°C. The capillary voltage was set at 3.0 kV. Cone voltage and collision energy were adjusted to optimize the signal of the most abundant product transition for guvacine: m/z 127.9 > 127.9 and the two most abundant product transitions for guvacoline: m/z 142.0 > 142.0 and 142.0 > 81.0, for arecoline m/z 155.7 > 43.8 and 155.7 > 155.7, for arecaidine m/z 141.7 > 43.8 and 141.7 > 141.7 and for nicotine d4 m/z 167.1 > 136.0. MassLynx 4.1 software was used for quantification.

Validation method: UPLC-MS/MS

Validation method was performed by UPLC-MS/MS because it is the method of choice for quantification. A six-point calibration curve ($n = 3$) was obtained by spiking blank hair at limit of quantification (LOQ) to 20 ng/mg at six different concentrations for each alkaloids. The LOQ was defined as the first point of calibration. The limit of detection (LOD) was the lowest concentration of analyte that could be detected with signal to noise ratio superior than 3. For the repeatability, two calibration points at 100 and 10 000 pg/mg were evaluated eight times. Recovery was not established as the method involves simple methanol incubation with no extraction step. The absolute extraction efficiency cannot be established, due to a lack of authentic reference material. Finally, matrix effect was established using 12 blank hair specimens spiked with the four alkaloids at 1 ng/mg.

Results and discussion

Arecoline, guvacoline, arecaidine and guvacine have very similar chemical structures. The UPLC-q-ToF-MS system enables to separate four alkaloids with exact mass and affinity property with the column, but the UPLC-MS/MS system is more suitable for quantifying the alkaloids in hair. For these reasons, the authors have used two methods to analyze accurately the four alkaloids in hair.

Ultra-high-performance liquid chromatography system coupled to time-of-flight mass spectrometry

The first step is the targeted screening. The UPLC-q-ToF-MS system allows the identification of the four alkaloids of betel nuts in hair and a specific library was created for this project.

The molecules were identified by their exact mass with an observed mass error (= (accurate mass/exact mass) \times 10⁶) from -1.4 to 1.6 ppm, from -3.4 to 2 ppm, from -5.9 to 3.5 ppm and from -17.4 to 8.1 ppm for arecoline, guvacoline, arecaidine and guvacine, respectively. Mass errors were considered satisfactory.

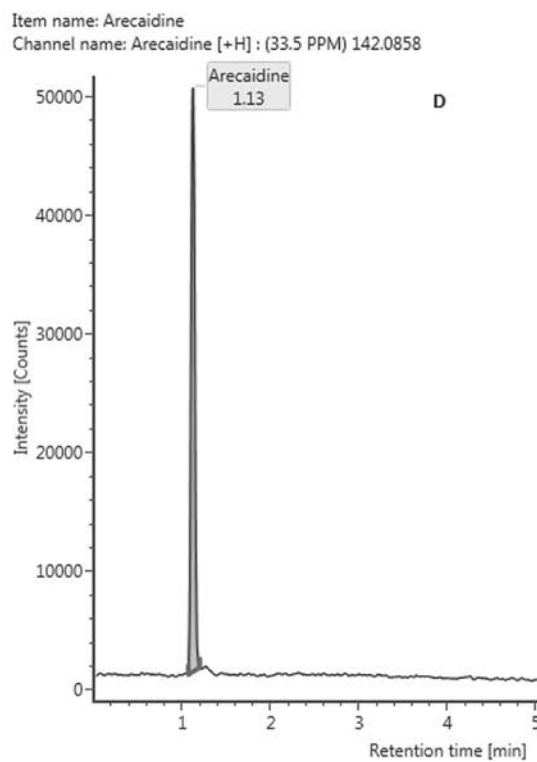
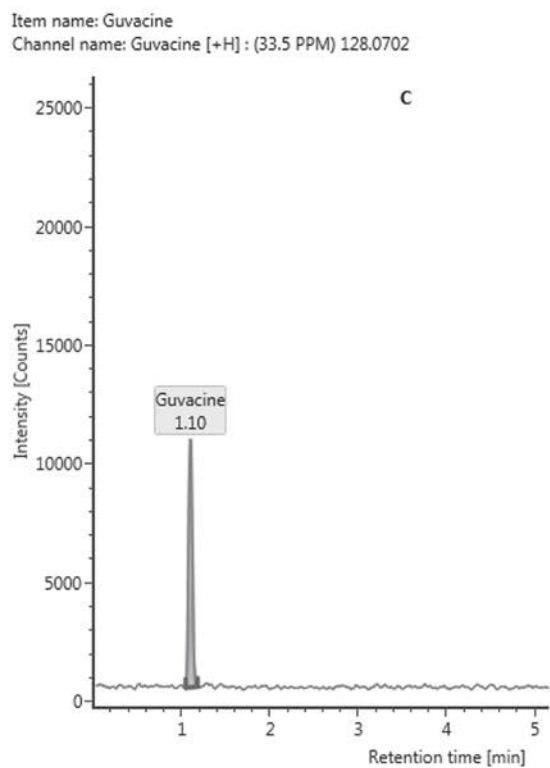
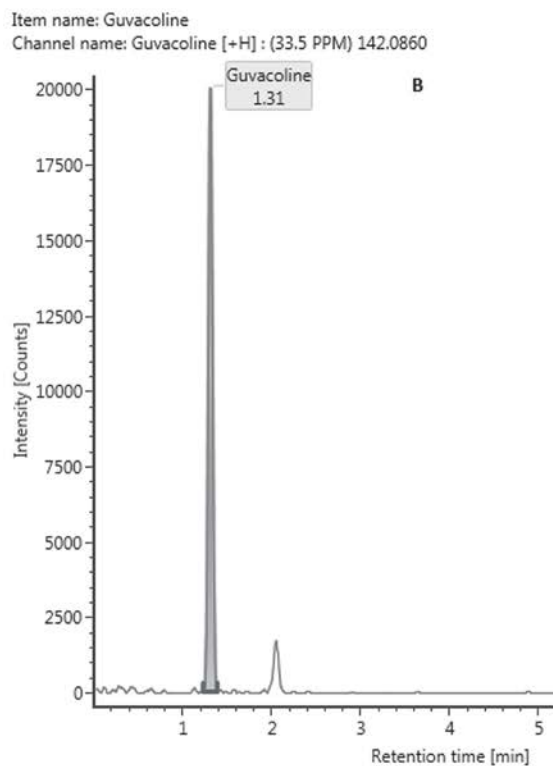
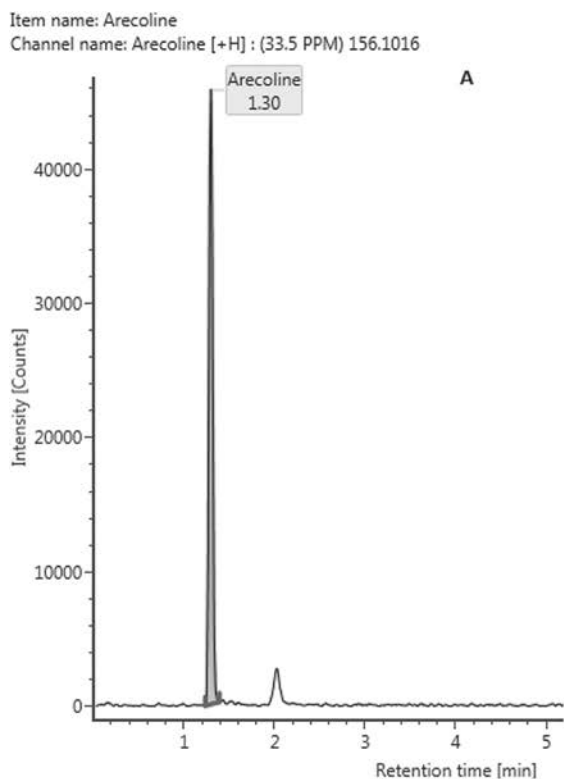


Figure 2. Chromatograms obtained from UPLC-qTOF-MS (sample 3): A, arecoline; B, guvacoline; C, guvacine; D, arecaidine.

The blank hair specimens were negative. A typical chromatogram, obtained after extraction of sample 3, is presented (Figure 2). Arecoline was identified in all samples. Guvacoline was identified in 16 samples and for 3 samples the identification was

described as tentative. Arecaidine has been identified in 13 samples, 2 were tentative and 4 were negative. Finally, guvacine was identified in 4 samples, while 12 were tentative and 3 were negative (Table II).

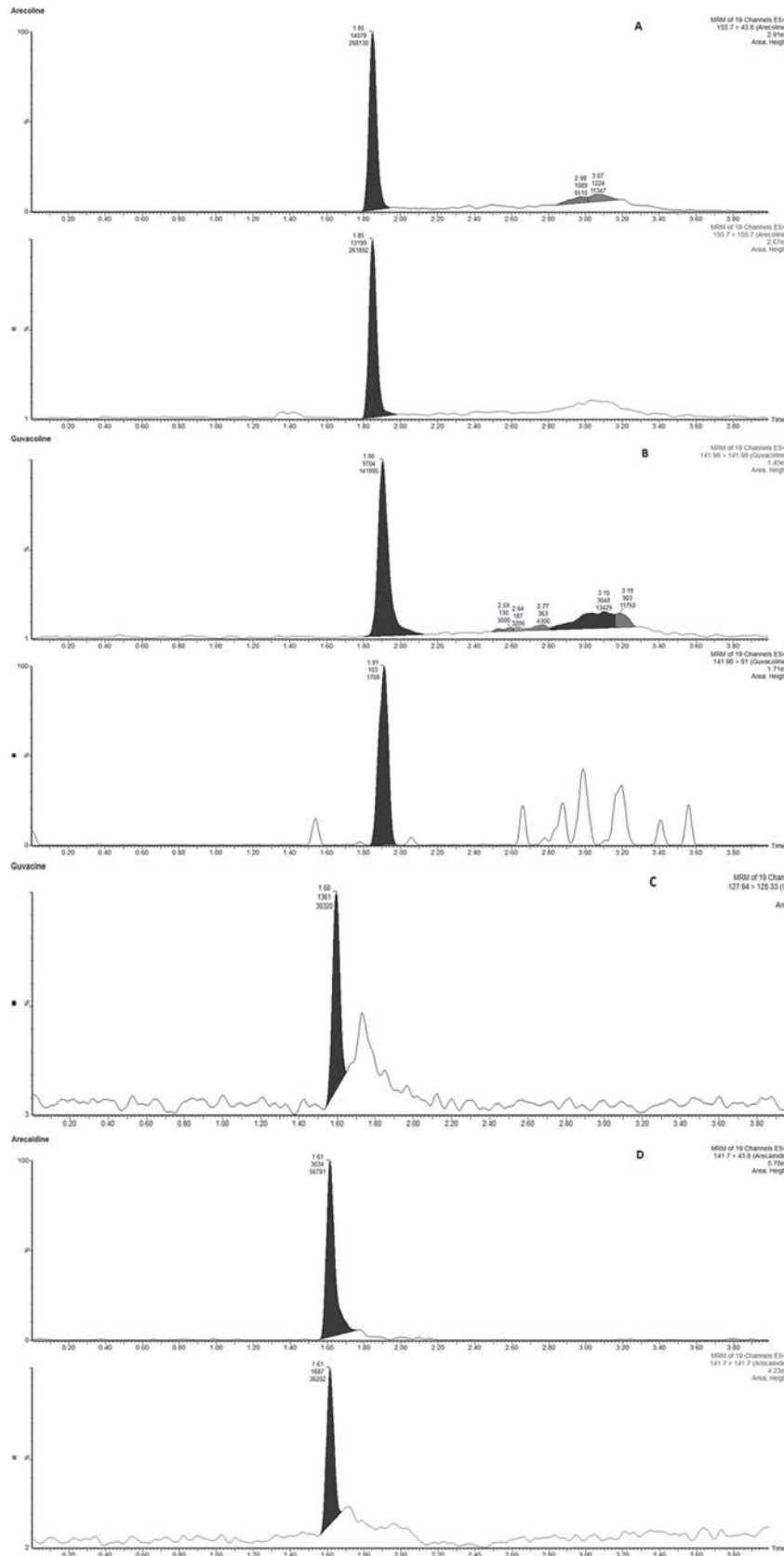


Figure 3. Chromatograms obtained from UPLC-MS/MS (sample 3). Concentrations were as follows: A, 1752 pg/mg arecoline; B, 125 pg/mg guvacoline; C, 437 pg/mg guvacine; D, 128 pg/mg arecaidine.

Table II. Results of Hair Samples

Sample	Arecoline (pg/mg)		Arecaidine (pg/mg)		Guvacoline (pg/mg)		Guvacine (pg/mg)		Ratio Arecaidine/ Arecoline	Ratio Guvacine/ Guvacoline	Ratio Arecoline + Arecaidine/ Guvacoline + Guvacine)
	UPLC-q- TOF	LC-MS/ MS	UPLC-q- TOF	LC-MS/ MS	UPLC-q- TOF	LC-MS/ MS	UPLC-q- TOF	LC-MS/ MS			
1	+	298	+	NQ	+	234	?	100	NA	0,43	0,89
2	+	483	+	NQ	?	ND	?	146	NA	NA	3,31
3	+	1752	+	128	+	125	?	437	0,07	3,5	3,35
4	+	143	-	NQ	+	ND	-	NQ	NA	NA	NA
5	+	2244	+	414	+	2095	+	182	0,18	0,09	1,17
6	+	3932	+	695	+	2548	?	159	0,18	0,06	1,71
7	+	2993	+	113	+	182	?	ND	0,04	NA	17,07
8	+	18178	+	3765	+	993	+	3186	0,21	3,21	5,25
9	+	1930	+	215	+	29	+	ND	0,11	NA	73,97
10	+	56	?	ND	?	ND	-	NQ	NA	NA	NA
11	+	15903	+	170	+	203	?	ND	0,01	NA	79,18
12	+	654	-	NQ	+	ND	?	ND	NA	NA	NA
13	+	3125	+	63	+	156	?	ND	0,02	NA	20,44
14	+	1073	-	NQ	+	ND	?	ND	NA	NA	NA
15	+	1283	+	67	+	ND	+	ND	0,05	NA	NA
16	+	3762	-	161	+	646	-	ND	0,04	NA	6,07
17	+	2787	+	NQ	+	14	?	ND	NA	NA	199,07
18	+	3982	+	NQ	+	ND	?	ND	NA	NA	NA
19	+	3057	+	189	+	ND	?	ND	0,06	NA	NA
Range		56-18178		63-3765		14-2548		100-3186			
Mean		3560		544		657		702			
Median		2244		170		203		170,5			

+, positive; -, negative; T, tentative; ND, not detected; NQ, not quantified; NA, not applicable

Table III. Validation Data

Analyte	Calibration curve			Sensitivity	
	Range (pg/mg)	Equation	R ²	LOD (pg/mg)	LOQ (pg/mg)
Arecoline	10-20 000	$y = 0.00003x - 0.0039$	0.9998	5	10
Guvacoline	10-20 000	$y = 0.00005x + 0.2904$	0.9981	5	10
Arecaidine	30-20 000	$y = 0.00005x + 0.009$	0.9998	10	30
Guvacine	50-20 000	$y = 0.00006x + 0.0679$	0.9999	10	50

Ultra-high-performance liquid chromatography system coupled to tandem mass spectrometry

Three calibration curves based on analyte/internal standard peak area ratios were obtained on three separate days and analyzed by weighted linear regression to validate the linearity of the method for each alkaloid.

For the four alkaloids, the assays were linear. Results of validation are presented in Table III. A typical standard curve of a validation assay, after weighted linear regression $1/x$, was obtained with a good correlation coefficient for each alkaloid where x represents the mass and y is the peak area ratio of arecoline to IS. The concentrations are calculated with the exact weighed mass.

QC samples (100 pg/mg and 10 000 pg/mg), analyzed in duplicate in eight independent experimental assay, were used to determine a coefficient of variation (CV) for precision and accuracy. These CVs

were lower than 20%. Irrespective of the drug, matrix effects were lower than 30%.

After identification using UPLC-q-ToF-MS, samples were quantified with UPLC-MS/MS system. The blank hair specimens were negative and the combined decontamination solutions were always negative. Table II presents all the results including quantification and drug ratios for parent drug versus hydrolyzed compound.

Measured concentrations were in the range 60 pg/mg to 18 ng/mg for arecoline ($n = 19$, mean = 3.5 ng/mg, median = 2.2 ng/mg), 14 pg/mg to 2.5 ng/mg for guvacoline ($n = 11$, mean = 657 pg/mg, median = 203 pg/mg), 63 pg/mg to 3.8 ng/mg for arecaidine ($n = 11$, 544 pg/mg, median = 170 pg/mg) and 100 pg/mg to 3.2 ng/mg for guvacine ($n = 6$, mean = 702 pg/mg, median = 171 pg/mg) (Table II). A typical chromatogram, obtained after extraction of sample 3, is presented (Figure 3).

The concentrations of arecoline (60 pg/mg to 18 ng/mg) are in accordance with those from the literature, as Marchei *et al.* (14) reported concentrations ranging from 300 pg/mg to 1.70 ng/mg.

Concentrations of guvacoline, arecaidine and guvacine are of the same order of magnitude as concentrations of arecoline. In 2010, Wu *et al.* (25) published that there was no correlation between alkaloids concentration in blood and the habits of betel nut consumption (frequency, duration of chewing, circulating drug concentrations). The dispersion of results in hair can be explained with the same phenomenon than blood as the plasma AUC (Area Under the Curve) is one of the parameters that influence drug hair incorporation.

Furthermore, it is to highlight that concentrations of guvacine can be higher than guvacoline in hair. Indeed, the arecaidine/arecoline ratios were varied from 0.01 to 0.18 and the guvacine/guvacoline ratios were varied from 0.06 to 3.50 (Table II). There is no correlation between the concentrations of hydrolyzed molecules and those of parent molecules. When possible, the ratio (Arecoline + Arecaidine)/(Guvacoline + Guvacine) were established and ranged from 0.89 to 199.07 (Table II). The absence of correlation can be explained by two reasons. First, the consumption habits with the use of lime in different proportions can modify the amount of hydrolyzed molecules. Indeed, the use of lime (basic condition) promotes ester hydrolysis (7–8). In addition, the variable composition of areca nut can also explain it. Most of the authors have published that arecoline is the predominant alkaloid in areca nut. For example, Wang *et al.* (26) indicated a composition of 7.5 mg/g for arecoline, 2.9 mg/g for guvacine, 2.0 mg/g for guvacoline and 1.5 mg/g for arecaidine. On the opposite, Jain *et al.* (23) published a different composition of the four alkaloids, i.e., guvacine (1.39–8.16 mg/g), arecoline (0.64–2.22 mg/g), arecaidine (0.14–1.70 mg/g) and guvacoline (0.17–0.99 mg/g).

Testing for betel nut alkaloids in hair enables to document long-term exposure. This analysis can be useful to make connections with some diseases such as cancer of the mouth but also track the consumption of pregnant women and prevent them from potential dangers for their children. For example, Pichini *et al.* (15) described concentrations of arecoline ranging 0.006–0.008 µg/g in meconium from exposed newborn, confirming the clinical outcomes observed for these newborns with low weight and hypotonia.

Conclusion

The use of two analytical methods allows to cross results and to identify accurately active ingredients of betel nuts in human hair. This study shows that it is necessary to analyze the four alkaloids because of the lack of correlation between hydrolyzed and parent molecules. The identification of these drugs in hair is a good marker of long-term consumption of betel nut, and allows us to document a local practice that remains difficult to evaluate just by questioning. The clinical significance of the findings remains to be established.

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