Published in final edited form as:

Subst Use Misuse. 2020 ; 55(9): 1395–1402. doi:10.1080/10826084.2019.1630442.

Chemical Markers for Short- and Long-Term Areca Nut Exposure

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Abstract

Background: Areca nut (AN) chewing causes oral cancer. AN cessation programs are the most effective approach to reduce AN chewing induced cancers but require biomarkers to determine program compliance and success.

Objectives: To explore chemical markers for short- and long-term AN exposure using non-invasively collected saliva, buccal cells (BCs), and scalp hair of chewers.

Methods: Saliva was collected from a male chewer before and up to 2 days after AN chewing. Saliva was separated into supernatant and pellet (buccal cells) then analyzed by spectrophotometry and liquid chromatography (LC) with UV/VIS detection. Scalp hair was collected from 4 chewers and analyzed for areca alkaloids using direct analysis in real time-tandem mass spectrometry (DART-MSMS).

Results: The red pigmented saliva after chewing showed no valuable signals when either the saliva supernatant or pellet (BCs) were analyzed by spectrophotometry. Saliva analysis by LC-UV/VIS showed diagnostically valuable signals at 488 nm up to 5 and 24 hours post chewing in the supernatant and pellet, respectively. DART-MSMS analysis detected 2 of the 4 AN specific alkaloids (arecoline and arecaidine) in male but none in female hair.

Conclusions/Importance: LC-UV/VIS analysis of the red pigments extracted from saliva and BCs after AN chewing showed distinct signals up to 24 hours post chewing while DART-MSMS analysis of in BCs and scalp hair showed selective signals of AN alkaloids for several weeks or months after AN exposure. Chemical hair treatment might prevent detection of areca alkaloids in hair. AN cessation trials and other programs now have essential tools for bioverification.

Keywords

areca nut; saliva; buccal cells; scalp hair; biomarkers; cessation program; HPLC; UV/VIS spectrophotometry; DART-MS

INTRODUCTION

Areca nut (AN) chewing is a carcinogenic habit practiced by an estimated 600 million people worldwide (Gupta & Warnakulasuriya, 2002) and is especially prevalent in areas of the western pacific region such as Guam where it is chewed alone or as a ‘quid’, a

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Disclosure statement: The authors declare no potential conflicts of interest

combination of the nut with betel (*Piper betle* L.) leaf and slaked lime (calcium hydroxide) with occasional additions of tobacco and/or other flavoring agents (IARC, 2004). The term AN will be used throughout to indicate the areca nut or its preparations.

AN chewing has been linked to increased oral cancer risk with tumor formation, typically squamous cell carcinomas, occurring at the nut-retained site in the mouth (Thomas & MacLennan, 1992; Trivedy et al., 2002). It is there where the AN -and particularly slaked lime- alkalizes the oral cavity and provokes the oxidation of AN polyphenols, which generates reactive oxygen species implicated in carcinogenesis (Nair et al., 1992). This reaction also produces a bright red pigmented polyphenolic polymer that darkens over time to a dark red to brown-blackish stain (Nair et al., 1992). Accordingly, chewing AN with slaked lime stains the saliva and the entire oral cavity initially a bright red pigment, which darkens over time due to the formation of secondary (oxidative) reaction products (Mathew, 1971). The initial and secondary red pigmented products have been previously characterized *in vitro* (Mathew, 1971), however, to our knowledge the red pigmented compounds produced *in vivo* have not been measured. The oral cavity is constantly exposed to AN during chewing. Thus, we hypothesize that the red pigmented compounds in saliva are adsorbed onto and/or absorbed into the oral mucosal buccal cells (BCs) of chewers where they can serve as short-term chemical markers of AN exposure (Franke et al., 2016).

The 4 areca alkaloids arecoline, guvacoline, arecaine, and guvacine are specific to the AN (Arjungi, 1976; IARC, 2004; Franke et al., 2015; Franke et al., 2016; Franke et al., 2019a) and can, therefore, serve as excellent and unique chemical markers for AN exposure. We previously used high performance liquid chromatography (HPLC) with mass spectrometry (MS) to quantify these areca alkaloids in saliva and BCs of chewers in Guam who chewed different AN preparations (Franke et al., 2015) and in saliva, BCs, and urine from chewers in Hawai'i (Franke et al., 2016; Franke et al., 2019b) up to several hours or days post chewing. However, biomarkers reflecting lengthier time periods are needed to validate long-term compliance to AN cessation trials, the single most effective approach to reduce AN-induced oral cancers. Biomarkers can serve to validate -and perhaps avoid- total reliance on commonly used self-reports. Despite its advantages, self-reported data can be unreliable, particularly when taken from subjects in an intervention program who feel an expectation to cease intake (Patrick et al., 1994).

Hair is a keratinous matrix that accumulates substances over time (weeks to months depending on the hair length) and, for this reason, has been used to document long-term drug intake (Kintz et al., 2006; Gallardo & Queiroz, 2008; Cappelle et al., 2015). However, we are unaware of its use to document long-term AN exposure. Hair analysis can be particularly useful when compounds are incorporated (exclusively) by consumption such as through chewing, rather than from sweat/sebum or external contamination (Gallardo & Queiroz, 2008) due to difficulties in distinguishing between the latter two and the former mechanisms (Pichini et al., 1996). Scalp hair from the vertex posterior region has the least variability in growth rate compared to other regions (Kintz et al., 2006) averaging around 1 cm/month (Society of Hair Testing, 2004; Gallardo & Queiroz, 2008) and is, therefore, commonly used to monitor temporal drug intake (Kintz et al., 2006; Duivivier et al., 2016). Segmented hair analysis can, therefore, be used to establish a time-course exposure.

Duvivier et al. recently applied a relatively new and sensitive ionization technique (direct analysis in real time, ‘DART’) to longitudinally scan and analyze via MS locks of hair from cannabis (Duvivier et al., 2014) and multiple drug (IARC, 2004; Duvivier et al., 2016) users with no sample preparation. When coupled to a mass spectrometer, DART-MS is capable of analyzing materials in their native form in the open air at ambient conditions without or with minimal sample preparation (Cody et al., 2005). This avoids the need for the time-consuming extractions and chromatographic separations required for HPLC. This also reduces analyte loss, sample consumption, and turn-around time and entirely avoids carry-over.

This study aimed to explore chemical markers using a variety of techniques including DART–MS analysis for short- and long-term AN exposure using saliva, BCs, and scalp hair of AN chewers.

METHODS

Chemicals and Materials

Ethylacetate (EtOAc), methanol (MeOH), hydrochloric acid (HCL), ethylenediaminetetracetic acid (EDTA), sodium chloride (NaCL), tris buffer, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Hampton, NH). Ammonium bicarbonate, (+)-catechin, and trichloroacetic acid (TCA), arecoline hydrobromide, and guvacine hydrochloride were purchased from Sigma Aldrich (St. Louis, MO). Sodium deoxycholate (SDC) was purchased from Thermo Scientific (San Jose, CA). Guvacoline hydrobromide, N-nitrosoguvacoline, arecaidine hydrobromide, and arecodine-d₅, hydrobromide salt were purchased from Medical Isotopes (Pelham, NH). AN, betel leaves and slaked lime were purchased from a local grocery store in Honolulu.

Preparation of AN extract

One ripe AN (husk included) was sliced longitudinally in half. One half was ground manually with 0.1g slaked lime, ¼ betel leaf, and 5 mL deionized water using a mortar and pestle for 11 mins to simulate chewing. The resulting slurry was filtered using a 0.22 µm vacuum filter unit for 20 hrs. One mL of the filtrate was acidified with 250 µL TCA, extracted twice with 1 mL EtOAc, dried under nitrogen flow, then reconstituted with 1 mL 5 mM ammonium bicarbonate in 10 mL MeOH. Ripe ANs, betel leaves, and slaked lime were obtained at a local vendor in Honolulu, Hawaii and kept cool until use.

Saliva collection

On several occasions a healthy adult male (infrequent chewer) donated saliva in Hawaii before and after chewing AN with slaked lime and betel leaf. The study participant refrained from eating or drinking for 30 minutes prior to chewing and, if food was consumed, brushed his teeth without tooth paste no less than 10 minutes before donating baseline (and consecutive) samples. Following the baseline saliva collection, the participant chewed for 5 minutes 1 green AN with 200 mg slaked lime wrapped in half a betel leaf (all obtained fresh from a local market in Hawaii) by moving it evenly in the entire mouth during chewing. Subsequently, saliva samples were collected at 0 (immediately after removing the AN from

the mouth), 0.5, 1, 2, 4, 8, 24, and 48, hours post chewing. Aliquots from each timed collection were stored immediately at -80°C . The IRB board from the University of Hawaii approved the collection of these samples and the participant signed an informed consent prior to study commencement.

Hair collection from chewers

Scalp hair samples were donated from 4 adult chewers (3 males, 1 females) in Hawai'i with naturally black hair. The volunteers self-reported chewing 4-10 times per day with 0.5-1 AN per chew. All chewers included lime and tobacco in their preparation. One male has been chewing for 6 years while the other 3 volunteers reported chewing 15-20 years. None has attempted to cease chewing. For negative control, scalp hair samples were donated from an adult non-chewer with naturally black hair. The IRB board from the University of Hawaii approved the collection of these samples and each participant signed an informed consent prior to study commencement.

Hair sample preparation for DART-MSMS analysis.

For DART analysis, similar to Duvivier et al. (Duvivier et al., 2014; Duvivier et al., 2016) tufts of hair (approximately 1~3 cm long) were attached to stainless steel IonSense open spot mesh screens (IonSense LLC, Saugus, MA) using adhesive tape at both ends. Longer length single hair samples (5~10 cm) were coiled and folded onto themselves to sizes of approximately 3 cm and taped onto the mesh screen. The mesh screen was held manually between the DART source outlet and the MS inlet. Alkaloid standards were prepared in MeOH in the range of 1 ng/mL to 1000 ng/mL. Following analysis of blank samples (MeOH), each liquid standard was loaded into a glass capillary, which was manually held between the source outlet and the MS inlet for 15~30 seconds every minute until there was no more liquid in the glass capillary. Following the standards, another blank was analyzed. These analyses were followed by the negative control (hair from a non-chewer) and subsequently hair from the 4 chewers.

Because the standards and hair samples are not the same matrix, the assay sensitivity using hair as matrix could not be determined unlike Duvivier et al (Duvivier et al., 2014; Duvivier et al., 2016), who externally spiked blank hair samples and mesh with a TLC nebulizer. However, the use of both a positive (liquid external standard) and negative (MeOH) control for the DART analysis enabled us to approximate the detection limit for areca alkaloids. Additionally, using a negative matrix control (hair from a non-chewer) permitted qualitative determination of alkaloids in the chewers' hair.

DART-MSMS settings

A DART-SVP ion source (IonSense LLC, Saugus, MA) was coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher, San Jose, CA). The standards or samples were placed between the source and MS for 15-30 seconds every minute for a total of 4 minutes, using one full scan followed by three multiple reaction monitoring (MRM) scans. The DART-SVP was operated in positive-ion mode with a temperature setting at 250°C , using high purity helium during target analyses and nitrogen during other times, both at 80 PSI. The mass spectrometer was operated with a capillary temperature at 350°C . Full

scan was performed with a scan range of m/z 50~200. MRM scans was performed with a scan width 0.5, scan time 0.1 sec and peak width 0.5. Collision energy was set at 15. Based on experiments with authentic standards the following mass transitions were used to detect selectively the target analytes (m/z of molecular ion): arecoline (156 $\rightarrow$ 44, 113); guvacoline (142 $\rightarrow$ 81, 113); arecaidine (142 $\rightarrow$ 44, 99); guvacine (128 $\rightarrow$ 99, 110).

Preparation of chewer's saliva samples for spectrophotometric and HPLC analyses

950 μ L thawed saliva was centrifuged at $1,000 \times g$ for 15 minutes to separate the specimen into liquid saliva (supernatant) and saliva pellet (precipitate). The supernatant was acidified with 2% TCA to pH 2 followed by extraction twice with 1 mL EtOAc. The organic phase was dried under a stream of nitrogen then re-dissolved in 150 μ L 2 mM NaOH for spectrophotometric and HPLC analyses of polyphenolic pigments. The remaining saliva pellet (containing sloughed off BCs and cell debris) was lysed by incubating the pellet at 37°C with 700 μ L lysis buffer consisting of 10 mM Tris buffer (pH 8), 10 mM EDTA (pH 8), 0.1 M NaCl, and 2% SDC. After 2 hours, 2% TCA was used to adjust the lysed pellet solution to pH 2 followed by extraction twice with 1 mL EtOAc. The organic phase was dried under a stream of nitrogen and then re-dissolved in 150 μ L 2 mM NaOH for spectrophotometric and HPLC analyses of polyphenolic pigments.

Ultraviolet-visible (UV/VIS) spectrophotometric analysis of chewer's saliva

Samples were placed in a 1 cm micro quartz cuvette then monitored at multiple wavelengths using a Shimadzu UV/VIS-1800 spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Maximal absorbance was observed at 488 nm.

HPLC analysis of chewer's saliva

HPLC analysis was carried out on a model Accela ultra HPLC system (Thermo Fisher, San Jose, CA) consisting of a Kinetex C18 column (150 $\times$ 3 mm, 2.6 μ m) coupled to a UV/VIS photo diode array detector (Thermo Scientific, San Jose, CA). Gradient elution was performed at a flow rate of 250 μ L/minute using a linear gradient with MeOH (A) against 5mM ammonium bicarbonate in water (B) starting at 20% A, holding for 1 minute then linear gradient to 60% A over 13 minutes linear gradient, back to 20% A over 2 minutes, then holding for 8 minutes for equilibration. Detection was performed from 300-600 nm and data were predominantly extracted from the 488 nm trace.

RESULTS

Generation and spectrophotometric analysis of red pigments from AN extract *in vitro*

Red pigments were generated *in vitro* by alkalizing the AN extracts to pH 11, as would occur when adding slaked lime; the color changed to light yellow by acidifying the extract with HCl to pH 2 (Figure 1). While this process was reversible, if left at high pH at room or higher temperature not protected from air, the red pigmented AN extract would change to dark red and later to brown-blackish in an irreversible manner over time due to the formation of secondary reaction products (Mathew, 1971). This showed that our *in vitro* generated red pigment behaved similarly to what would be expected *in vivo*.

When analyzed spectrophotometrically, the absorbance spectrum of the AN extract shifted, as expected, depending on the pH (acidic, neutral, alkaline). Absorbance was highest at 488 nm when in an alkaline milieu (pH>11), which is similar to (+)-catechin and to that found by Mathew, 1971 (Mathew, 1971) (data not shown). From this, we suspected the red pigments were polyphenols and similar in structure to catechin, a main polyphenol in AN (Mathew, 1971).

Spectrophotometric analysis of red pigments from liquid saliva and saliva pellets of chewers *in vivo*

Red pigments generated *in vivo* by the chewer after AN chewing did not show any valuable UV/VIS signals when either the liquid saliva (supernatant) or saliva pellet (which contained sloughed off BCs) was analyzed (data not shown) by spectrophotometry. Concentrating the extract did not resolve this issue. From this, we concluded that UV/VIS absorption by spectrophotometry was not sensitive enough to measure the low concentrated red pigmented compounds.

HPLC-UV/VIS analysis of red pigments from liquid saliva and saliva pellets of chewers *in vivo*

Using HPLC with UV/VIS detection, we were able to identify diagnostically valuable signals at 488 nm up to 3-5 hours post chewing in the saliva supernatant and up to 24 hours post chewing in the saliva pellet (Figure 2). Due to the much longer detection signals in the saliva pellet, which contains sloughed off BCs, we chose to isolate and analyze exclusively BCs in subsequent experiments with the hypothesis that AN-specific chemicals adhere (mostly) to the outer layer of the BCs.

DART-MSMS analysis of areca alkaloids from hair of chewers

We recently applied DART-MS in high resolution mode successfully to quantitate arecoline and arecaidine-guvacoline from BCs exposed *ex vivo* to AN extracts (nut, leaf, lime, and tobacco) up to 7 days post exposure and from BCs of AN chewers up to 3 days post chewing (Franke et al., 2019b). Due to these exciting findings and those from Duvivier (Duvivier et al., 2014; Duvivier et al., 2016) who successfully applied DART-MS to analyze multiple drugs from hair, we applied DART-MSMS in a creative approach to analyze areca alkaloids in scalp hair samples from 4 adult AN chewers (3 males, 1 female) to include exposure changes over longer time periods. MRM scans qualitatively showed the presence of all 4 areca alkaloids standards; however, sensitivities as measured from authentic standards were found to be vastly different with arecoline at 100%, and arecaidine at 10.1% while guvacoline and guvacine were only 1.1% and 0.7% intensities, respectively (Figure 3A). No areca alkaloids signals were present during the entire run time in the negative control (non-chewer) hair sample (data not shown). Among the chewers, exclusively arecoline and arecaidine were found in the male but not the female hair samples (Figure 3B).

DISCUSSION

AN chewing cessation is the single most effective approach to reduce AN chewing induced oral cancers. For this reason, cessation programs such as the Betel Nut Intervention Trial

("BENIT"; trial) #NCT02942745) has been recently implemented in Guam and Saipan. The BENIT is the first known randomized intervention trial in the western pacific region aimed at ceasing AN chewing. However, the BENIT and other cessation programs like it urgently require measurable biomarkers that reflect AN chewing to determine short- and long-term program compliance in order to evaluate their success. The current study explored chemical markers to fulfill this need.

In this study, we first sought to analyze the red pigments generated by AN preparations in the saliva of chewers. Following centrifugation, analyses of the saliva pellets using HPLC-UV/VIS showed distinct peaks up to 24 hours post AN exposure when monitored at 488 nm (Figure 2). This was longer than the signals found in the saliva supernatant (3-5 hours post exposure; figure 2) and more sensitive than UV/VIS spectrophotometry analysis, which did not detect any valuable signals. However, a biomarker present for only 24 hours post exposure would not be helpful in monitoring compliance to a cessation trial where follow-up typically occurs several months following intervention termination. Then again, biomarkers representing AN exposure for several days, while relatively short, could be important in reflecting short-term compliance before or during intervention programs, such as would occur during washout or run-in periods, elapsed time periods during which drugs or treatment effects are eliminated from the body. These are crucial times when compliance to cessation is imperative to determine the overall treatment effect. Thus, we pursued a short-term biomarker to represent several days AN exposure.

Due to the longer lasting signals in the saliva pellet (containing sloughed off BCs) versus supernatant, we chose to isolate and analyze exclusively BCs in subsequent experiments. One such experiment used DART-MS to analyze human BCs exposed *ex vivo* to AN extracts and BCs obtained from chewers before and after AN chewing. DART ionization and measurement by orbitrap based MS showed signals for areca alkaloids arecoline and arecaidine-guvacoline from BCs exposed *ex vivo* up to 7 days after AN extract exposure and from BCs of chewers up to 3 days post chewing, the last time periods tested (Franke et al., 2019b). Arecaidine and guvacoline are isomeric and, therefore, have identical masses. Thus, these compounds could not be separated by orbitrap MS alone, which detects unfragmented parent molecular masses. Applying the more sensitive tandem MS (MSMS) detection in future studies would allow differentiation between the two isomeric compounds.

In the current study, we used DART- MSMS to analyze areca alkaloids from scalp hair of AN chewers. Scalp hair is a non-invasively collected matrix widely used to reflect long-term drug intake (Kintz et al., 2006). MSMS detection is a sensitive, selective, and diagnostically valuable technique especially when used in selective ion monitoring or selective reaction monitoring (MRM) mode because it is able to differentiate isomers (such as arecaidine from its isomeric guvacoline) using their different fragmentation patterns. Using DART-MSMS in MRM mode, we were able to detect all 4 areca alkaloids in standards at concentrations as low as 0.1 ng/mL and could differentiate between isomers arecaidine and guvacoline (Figure 3A). In the male hair samples, both arecoline and arecaidine were prominently detected, while guvacoline and guvacine were not (Figure 3B). We think the main reason for this is that the measurement sensitivity by DART-MSMS is sufficient for arecoline and arecaidine but not for the other alkaloids due to the relative sensitivities as measured from authentic

standards being 100% for arecoline and 10.1%, for arecaidine, but only 1.1% for guvacoline and 0.7% for guvacine.

In 2014, Duvivier and colleagues conducted a feasibility study using DART with orbitrap based MS to longitudinally scan untreated locks of hair from cannabis users for tetrahydrocannabinol (THC) (Duvivier et al., 2014). The longitudinal scanning from the scalp to hair tip indicated their innovative hair scan method could be used to assess temporal drug intake with an accuracy of ± 2 weeks. Additionally, the authors detected THC in a sub-sample of hair decontaminated with dichloromethane, providing compelling evidence that the majority of detected THC was due to intake and not external contamination. In a subsequently study (Duvivier et al., 2016), the same authors again applied DART with orbitrap-based MS hair scan method for targeted and untargeted detection of multiple illicit drugs from known users and utilized full-scan and data-dependent product ions acquired from DART quadrupole-orbitrap MS to confirm compound identity.

We were unable to detect any alkaloids in the female hair sample. This could be due to chemical hair treatments or excessive use of hair products since all other factors regarding AN chewing habits were similar to the male volunteers. However, no information was collected from the female volunteer regarding hair treatments. Further studies with more female hair samples and information regarding chemical hair treatment are warranted to give evidence for our speculations. Melanin is a polyanionic compound known for retaining drugs in hair (Larsson, 1993) and substances with cationic properties have been shown to have a high affinity to bind with melanin (Larsson, 1993). However, use of common hair products such various shampoos can alter the anionic properties of hair which, in turn, can alter the structural and chemical properties of melanin thereby potentially affecting its binding/affinity to areca alkaloids (Gavazzoni Dias et al., 2014). Our small sample size ($n=4$ hair samples) precludes further conclusions and future studies using DART-MSMS to analyze scalp hair from more AN chewers (both females and males) are needed to confirm our preliminary findings.

CONCLUSION

HPLC-UV/VIS analysis of the red pigments generated during AN chewing extracted from saliva and BCs shows distinct signals at 488 nm up to 24 hours post chewing and, therefore, would be useful only as a very short-term biomarker tool. However, analysis of areca alkaloids in BCs and scalp hair by DART-MS and -MSMS was shown to be a reliable modality to obtain information on AN exposure for several days and weeks or months, respectively. AN cessation trials and other programs have now essential tools for bioverification. However, additional studies with larger sample numbers and a detailed quantitation approach are warranted to verify our preliminary qualitative hair analysis results with 4 volunteers.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Kevin Cassel (UHCC) and Srue Wakuk for assistance with hair sample collections, Tricia Debaun for assistance with HPLC and spectrophotometry analysis, and all volunteers for their specimen donations.

Funding: National Cancer Institute awards U54 CA143727 and P30 CA71789

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Figure 1.

Schematic diagram depicting the hypothesized molecular basis of pH dependent color changes of aqueous areca nut extracts. At low pH, phenols are yellow and non-ionic. At high pH, phenolates are formed which are red and ionic and, thus, not soluble in ethylacetate for extraction. The color change due to pH change is reversible initially, however, these red pigments irreversibly darken over time probably due to the formation of oxidative reaction products representing o-quinones and their polymers (Mathew, 1971).

Figure 2.

Traces from HPLC-UV/VIS analysis of EtOAc extracts of timed saliva samples collected from a chewer before, during, and up to 3 days after chewing half an areca nut with lime wrapped in a betel leaf (0 h = during chewing). At 488 nm, diagnostically valuable signals were observed up to 3-5 hours after chewing in the saliva supernatant extract (left panel) and up to 24 hours after chewing in the saliva pellet extract (right panel). Signals decreased in size from 0 hours to later time points (circled in black) and disappeared in noise after 3 hours in liquid saliva and after 24 hours in the saliva pellet (not shown).

Figure 3. DART-MSMS analysis in MRM mode A) from authentic areca alkaloid standards at 0.1 ng/mL concentration. Relative sensitivities are arecoline 100%, arecaidine 10.1%, guvacoiline 1.1% and guvacine 0.7%. B) from hair samples of male chewers.

When hair was held between the DART source and MS orifice during 4×15 -second measuring times specifically the 4 areca alkaloids (listed in left pane) were detected. Among all the 4 areca alkaloids monitored all 4 analytes were detected in standards but only arecoline and arecaidine show distinct signals (indicated by black arrows) in male hair samples. No areca alkaloids signals were present when hair was removed from the DART source (area between arrows) or during the entire run time in the negative control hair (data not shown) or in the female hair sample tested.