Systematic toxicological analysis of Indian herbal ready-to-chew pouches by gas chromatography mass spectrometry

Abstract – Objective: Systematic toxicological analysis by gas chromatography-mass spectrometry has been applied for the analysis of Indian herbal ready-to-chew pouches. Material and methods: These small packages containing a powder formed by areca nut slices, tobacco, menthol and lime, are available in traditional markets and web sites. Their consumption has been recently spread in the asiatic countries not only among adults but especially among young people. Acute and chronic side effects, an increase in the incidence of oral and esophagus cancer and the possibility to develop dependence and tolerance phenomena following repeated pouches consumption prompted the systematic toxicological analysis of gutka and panmasala pouches. Gas chromatography mass spectrometry was used to verify the presence of declared substances, and to investigate the possible presence of licit and illicit psychoactive compounds. Results: Arecoline (1.35–1.91 mg/g) and nicotine (0.31–0.71 mg/g) and arecoline alone (1.67–2.74 mg/g) were respectively identified in gutkha and panmasala pouches, together with menthol (1.89–2.78 mg/g gutkha and 1.91–2.84 mg/g panmasala). No other pharmacological active compounds were identified. Conclusion: The mg amounts of the two alkaloids found in the pouches together with the reported consumption of several pouches a day may be consistent with health risks.

Key words: Areca nut, Tobacco, pouches, Panmasala, gutkha
1 Introduction

The areca nut, the fruit of the Areca catechu tree of the ‘palmaceae’ family, is the fourth most commonly used drug in the world after caffeine, tobacco and alcohol [1]. It is commonly consumed by betel quid (folded leaf package of nut slices and cut tobacco) chewing or betel nut (a little cigarette formed by the same ingredients of quid) smoking by Asian populations both men and women, and Asian communities living in Europe and North America [1–3]. Having an ancient history, they are an integral part of the culture and sometimes erroneously believed to have medicinal benefits [4, 5].

Whereas betel nuts are mainly smoked by the adult male population, the most commonly used product is betel quid consumed also by women, young adults and teenagers. It consists of a spicy flavoured and sweetened dry mixture of areca nut, slaked lime (calcium hydroxide) with tobacco (gutkha) or without it (panmasala) [6, 7].

Betel quids with or without tobacco and areca nut have been classified as carcinogenic to humans [8]. Indeed, tobacco use (both by smoking or chewing) is a major risk factor for cancer, having caused 100 million deaths in the 20th century [8]. India also has one of the highest rates of oral cancer in the world, partly attributed to high prevalence of tobacco chewing [6, 9]. It has also been shown that regular chewers of betel leaf and areca nut have a higher risk of damaging their gums and acquiring cancer of the mouth, pharynx, esophagus and stomach [7, 10]. Studies have found that tobacco and caustic lime increase the risk of cancer from areca nut preparations [1, 7, 10].

In the last few decades small, attractive and cheap packages of betel quid substitutes (the so-called pouches) have become widely available, often claimed to be safer products. Promoted by a slick, high profile advertising campaign and aggressive marketing, pouches of panmasala and gutkha have become very popular within all sections of Indian society, including school children. For most children, teenagers and women, cigarette smoking still remains a taboo in India. Conversely, areca nut chewing enjoys social acceptance and is also popular among men and women as well as among teenagers. It has been noticed that young school and college children have become the victims of these packages; most of them start using more and more pouches a day as an experiment or due to the pressure from friends [6]. These alternative tobacco products are often advertised as being safer than conventional cigarettes, leading to a much higher frequency of use, so that these younger chewers constitute an alarming prevalence for a new epidemic of oral cancer [6]. In particular, young children start using sweetened areca nut products, often adding tobacco later in their adolescence [6].

In recent years, the Italian anti-adulteration and safety bureau (Carabinieri per la Tutela della Salute -NAS) and the Italian National Institute of Health started an investigation on national and international internet market of dietary supplements, “smart drugs”, legal highs, spice products of both natural and synthetic origin.

Within the framework of this investigation, the Italian National Institute of Health analyzed ready-to-chew pouches products purchased from Indian web-sites by Indian analytical toxicologists, never analyzed for their content before.

The aim was to assess the presence and the amount of nicotine and arecoline and investigate the possible undeclared presence of other natural and synthetic substances with activity on central nervous system. In this concern, a systematic toxicological analysis (STA) using a gas chromatography – mass spectrometry was applied to screen commercial betel quid products under investigation [11].

2 Materials and methods

2.1 Chemicals and materials

Standards of nicotine, arecoline, menthol and pilocarpine used as internal standard, N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were obtained from Sigma-Aldrich (Milano, Italy). All reagents were of analytical-reagent grade.

Fifty different commercial betel quid pouches, whose labels reported the presence (but not the content) of areca nut, catechu and lime with tobacco (gutkha, N=30) or without tobacco (panmasala N=20) purchased from different Internet web sites and naturalistic shops were obtained by Indian colleagues involved in the present study. Pouches presented as coloured plastic bags containing approximately 3 g brownish powder (Figure 1).

2.2 Preparation of standard solutions

Stock standard solutions (10 mg/mL) of analytes were prepared in methanol. Working solutions at concentrations of 1 and 0.1 mg/mL were prepared by dilution of the stock standards-with methanol, and stored at –20°C until analysis. The internal standard (I.S.) working solution was used at a concentration of 10 mg/mL.

Calibration standards containing from 0.05 to 4.0 mg of arecoline, nicotine and menthol per g powder, were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 100 mg of calcium hydroxide powder. Methanolic solutions were evaporated under nitrogen before adding calcium hydroxide. Quality control samples of 0.1 (low control), 1.5 (medium control) and 3.5 (high control) mg analytes per g calcium hydroxide powder were prepared and included in each analytical batch to check calibration, accuracy and precision of samples.

2.3 Sample preparation and extraction

Extraction of compounds under investigation was performed by liquid-liquid extraction (LLE) after suspending 100 mg of each product, added with 15 μL I.S. solution, in 2 mL...
0.1 M phosphate buffer at three different pH: acid (pH=2.5), basic (pH=10–12) and neutral (pH=7.0). The samples were placed in an ultrasonic bath for 15 min, and then the solutions were extracted twice with 3 mL chloroform/isopropanol (9:1, v:v). After centrifugation, the organic layer was divided into two 3 mL aliquots evaporated to dryness at 40°C under a nitrogen stream. The first dry aliquot was derivatized with 100 μL O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) at 70°C for 30 min. The second dry aliquot was dissolved in 100 μL ethyl acetate. Derivatization was mandatory to increase the volatility and thermal stability of the compounds for polar and thermolabile compounds to make them suitable to chromatographic analysis. The reduction in polarity could also improve the gas chromatographic properties of the compounds by minimizing the undesirable and non specific column adsorption and by allowing for better peak shape and reduction in appearance of ghost peaks.

A 1 μL amount of underivatized and derivatized acid basic and neutral extracts were injected into the GC-MS system.

**2.4 Instrumentation**

Gas chromatography–mass spectrometric (GC–MS) analyses were carried out on a 6 890 Series Plus gas chromatograph equipped with an Agilent 7 683 autosampler and coupled to a 5 973 N mass selective detector (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Agilent Chemstation, Palo Alto, CA, USA).

**2.5 GC–MS conditions**

Separation was achieved on a fused silica capillary column (HP-5MS, 30 m × 25 mm i.d., film thickness 0.25 μm) (Agilent Technologies, PaloAlto, CA, USA). The oven temperature was programmed at 100°C for 2 min and then increased to 290°C at 10°C/min. A chromatographic run was completed in 30 min, and afterwards initial conditions were restored in 5 min. Split injection mode (15:1) was used and helium (purity 99%), with a flow rate of 1 mL/min as carrier gas. The injection port, ion source, quadrupole, and interface temperatures were: 260, 230, 150 and 300°C, respectively. The electron-impact (EI) mass spectra were recorded in total ion monitoring mode (scan range 40–550 m/z) to determine retention times and characteristic mass fragments. The full-scan data files acquired by GC–MS system were screened for the presence of peaks and mass spectra of any declared and non declared substance by Data Analysis Agilent Chemstation software. A first manual screen of the total ion current (TIC) by experienced personnel was followed by computer assisted identification of eventual unknown compounds, using the mass spectra libraries (NIST, WILEY and W9N08) provided by GC-MS system manufacturer containing underivatized and derivatized forms of the principal drugs of abuse (opioids, cocaine, amphetamines, 3,4 methylenedioxide-derivatives of amphetamines, cannabinoids, dimethyltriptamine, ketamine), the most abused psychoactive compounds (benzodiazepines, synthetic cannabinoids, methylphenidate, fluoxetine, paroxetine, sertraline) and the most common active principle of herbal “smart drugs” (e.g. ephedra alkaloids, synephrine, yohimbine, ergine, kavaine). The acceptance criteria for substance identification was based on the selection of three qualifying m/z for selected ions intensity ratios was a deviation ≤20% of the average of the ion intensity ratios of all the calibrators.

Characteristic ions were m/z 156, 140, and 118 for arecoline, 163, 132 and 106 for nicotine and m/z 209, 96 and 95 for pilocarpine. The protonated molecular ions at m/z 156 for arecoline, m/z 163 for nicotine and m/z 209 for pilocarpine were selected for quantification.

**2.6 Validation procedures**

Prior to application to real samples, the method was tested in a 3-day validation protocol [12, 13]. Selectivity, carry over, matrix effect, recovery, linearity, limit of detection (LOD) and quantification (LOQ), precision and accuracy were assayed as previously reported [14, 15].
In brief, 100 mg calcium hydroxide powder added with 100 μL of 1 mg/mL methanolic mixture of principal drugs of abuse, the most abused psychoactive compounds and the most common active principle of herbal “smart drugs”, as reported in the previous section, were extracted and analyzed for assessment of potential interferences. The apparent responses at the retention times of the analytes under investigation were compared to the response of analytes at their lowest quantifiable concentration. The potential or carry over was investigated by injecting extracted calcium hydroxide powder, with added IS, immediately after analysis of the highest concentration point of the calibration curve on each of the days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analytes under investigation.

For an evaluation of matrix effects, the peak areas of extracted calcium hydroxide powder spiked with quality control samples after the extraction procedure were compared to the peak areas of methanolic standards using four replicates.

Analytical recoveries were calculated by comparing the peak areas of calcium hydroxide powder spiked with quality control samples prior to and after the extraction procedure at different pH using four replicates at each level.

Linearity was studied in the calibration curves range. Five calibration points were tested in triplicate using peak area ratios between compounds and I.S. for calculations. A weighted (1/concentration) least-squares regression analysis was used for slopes and intercepts (SPSS version 9.0.2 for Windows). Standard deviation of the mean noise level over the retention time window of each analyte was used to determine detection limit (LOD = 3SD) and the quantification limit (LOQ = 10SD). To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative standard deviation and relative error, respectively.

### Results and discussion

First of all, systematic toxicological analysis of the ready-to-chew pouches confirmed the presence of nicotine and arecoline together with menthol in the thirty gutkha samples and of arecoline with menthol in the twenty panmasala samples (Table 1). These three substances were quantified in the basic fraction of the liquid-liquid extraction.

As shown in Figure 2, no additional peaks due to substances such as the principal drugs of abuse (opioids, cocaine,

![Graph](image_url)
amphetamines, 3,4-methylenedioxyderivatives of amphetamines, cannabinoids, dimethyltryptamine, ketamine), the most abused psychoactive compounds (benzodiazepines, synthetic cannabinoids, methylphenidate, fluoxetine, paroxetine, sertraline) or the most common active principle of herbal “smart drugs” (e.g. ephedra alkaloids, synephrine, yohimbine, ergine, kavain) [16] could be identified in any of underivatized and derivatized acid basic and neutral extracts of the analyzed pouches.

Blank samples injected after the highest point of the calibration curve did not present any traces of carryover.

With respect to the matrix effect, the comparison between peak areas of analytes spiked in extracted blank samples versus those for pure diluted standards showed less than 10% analytical signal suppression.

The analytical recoveries obtained after liquid /liquid extraction at basic pH ranged between around 81.2 and 92.3% (with no more than 5% standard deviation between different concentration levels) for all the analytes under investigation. Lower recoveries and poor peak shapes were obtained at neutral and acid pH.

Linear calibration curves were obtained for the compounds of interest with a correlation coefficient (r) higher than 0.99 in all cases; limits of detection (0.01 mg/g for all the analytes) and quantification (0.05 mg/g for all the analytes) were considered adequate for the purposes of the present study.

Intra-assay and inter-assay precision and accuracy calculations show relative standard deviations and relative errors always below the 15% for all the analytes satisfactorily meeting the internationally established acceptance criteria.

Even though it was found that no psychoactive natural or synthetic substances other than arecoline and nicotine were present in the pouches, these product cannot be considered safe or without any health risks. The amount of nicotine and arecoline measured in the analyzed preparations are in the order of mg per gram.

Data on acute toxicity of arecoline have been reported only for animal models [16]. Nonetheless, considering that the analyzed pouches contained approximately 3 g powder and that the same users anecdotally report the intake of more than ten up to twenty pouches/day, non negligible quantity of the above reported alkaloids are daily consumed by adult and young users [16].

Indeed, many acute and chronic adverse effects have been reported following betel quid consumption. Not only cardiovascular pathologies, arterogenesis in the large blood vessels, depletion of vitamin B12, increase in the blood pressure but also a higher risk of developing squamous cell carcinoma as well as other types of tumors [16].

Furthermore, even not completely clear if the betel quid consumption is able to induce true dependence phenomena, following chronic use of betel, especially if associated to tobacco, a dependence very similar to the one observed with cigarette smoking can be developed [17]. Besides, people who stop chewing the betel can show episodes of reversible toxic psychoses characterized by hallucinations and maniacal ideas [18].

4 Conclusion

For the first time systematic toxicological analysis by GC-MS has been applied for the analysis of Indian herbal ready-to-chew pouches. Arecoline and nicotine and arecoline alone have been identified in gutkha and panmasala pouches, respectively with no other licit of illicit psychoactive substance. The mg amounts of the two alkaloids found in the pouches and the reported consumption of several pouches a day is consistent with health risks, as evidenced by different authors.

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References


