Brazilian Journal of Forensic Sciences, Medical Law and Bioethics

Journal homepage: www.ipebj.com.br/forensicjournal

Stanley and Acetolysis: Enhanced Method for Pollen Identification and Geolocation of Cocaine Samples

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Received 18 February 2022; Accepted 21 July 2022

Abstract. The concept of palynological assemblage, a set of all identifiable pollen grains and spores, is very useful in geolocation determinations in Forensic Studies. To identify plant genera, palynologists must recognize detailed aspects of apertures, ornamentation and layers on the cell wall. Pollen and spores recovered from higher purity cocaine samples represent an alternative to indicate drug source regions. After following the method established by Stanley in 1992, we added the acetolysis procedure in samples seized by the Civil Police of the State of Sao Paulo, Brazil, in 2017, in order to enhance morphological traits observation and taxonomical identification of different pollen taxa, allowing reliable environmental and geolocation reconstructions.

Keywords: Forensic palynology; Cocaine; Geolocation; Brazil; Drug trafficking.

1. Introduction

According to the United Nations Office of Drugs and Crime (UNODC) 2020 Report[1], Brazil is one of the largest members of cocaine trafficking flows of the world. Due to its geographic position and transport structure, Brazil plays a

http://dx.doi.org/10.17063/bjfs12(2)y20223105-113

strategic role as an exporting country, even though it is not a recognized coca producer. However, current forensic analyses raise a significant challenge to Brazilian Law Enforcement and to the Legal System, for basic analytical methods employed in cocaine chemical identification are not able to specify unequivocally drug origin.

Among worldwide important methods to evaluate chemical cocaine profiles to indicate possible sources and connections among samples are minor alkaloids, truxilines levels and isotope ratios[2,3,4]. Efforts in this direction occur through the actions of the Brazilian Federal Police in the PeQui Project (*Perfil Químico - Chemical Profile*). One of its expected results is to build databases containing chemical profiles from known geographical locations to aid in pointing out trends in drug trafficking origins and routes[5,6].

An alternative method, with potential for unambiguous determination of regional origin and routes traceability of cocaine, is given by palynological analysis, i.e. identification of pollen and spore grains, which due to specific morphological characters especially their minute sizes can be deposited and be trapped in different matrices[7,8].

These microscopic plant structures are specific to each botanical taxon and thus may be used to indicate geographical sources, since floristic compositions are unique to particular ecological and vegetation zones. In addition, pollen dispersal varies according to distinct pollination strategies such as wind-borne as opposed to animal vectors ranging from insects to birds and even bats and other small mammals[9]. The strategy of short distance transport, by insects, predominates in tropical areas where cocaine-producing countries are located, is therefore applicable to geolocation analyses.

Many kinds of plants growing next to cocaine shrubs belonging to *Erythroxylum coca* Lam., *Erythroxylum novogranatense* (D.Morris) Hieron. and its varieties in Colombia, Peru, Bolivia and possibly in a small area within the western Brazilian Amazon, can release large quantities of pollen grains and spores, which may be deposited over coca leaves. Another important source of pollen and even algal cysts or diatom frustules in cocaine samples is water used in the beginning of extraction procedures[10]. All these biological markers can be retained in the cocaine powder or paste during refining and may be carried on into brick-shaped compacted volumes for overseas or long distance

shipping. Plastic materials and tapes used in wrapping of cocaine bricks can also aggregate pollen indicative of transport routes and at the same time prevent contamination of samples used in forensic geolocation analyses.

One well established method to recover pollen grains and spores from cocaine samples was created by E. A. Stanley[11], from the New York Police Department, USA, which was used as a basis for this study. We analyzed 100 grams of cocaine hydrochloride powder, pure aspect*, from an 800 gram brick seized by the State of Sao Paulo Civil Police, in Brazil, then followed Stanley's method and indicate a significant improvement in pollen identification.

2. Methods

The method established by Stanley[11] to recover pollen and spores from cocaine samples is based on methanol extraction and centrifugation. Both are well known in many different practices carried out in forensic laboratories, besides the fact that this method is easily performed, and these aspects are fundamental when considering police facilities, infrastructure and training.

Due to its chemical characteristics, methanol acts as a cocaine solvent. This means that after centrifugation, the drug is suspended in it, as a unique phase solution, where pollen grains, spores and other debris become a separated phase at the bottom.

It is noteworthy to mention that the last step in Stanley's method[11] requires Safranin-O as a staining procedure to allow identification of many grains at only family level.

Our sample analysis was performed at the Seized Drug Laboratory of the Superintendence of the Technical-Scientific Police of the State of São Paulo, and completed it by changing the last step, adding the acetolysis procedure at the Institute of Geosciences of the University of Sao Paulo. Therefore, we propose the following method beginning with the sequence recommended by Stanley[11] and ending with acetolysis.

Firstly, we added 400 ml of methanol in a becker containing 100 grams of cocaine sample stirring until total mixing. This solution was then distributed in

* Previously, Gas chromatography - mass spectrometry analysis in our cocaine sample confirmed the absence of common adulterants, such as caffeine, lidocaine and acetaminophen (undisclosed information).

50 ml tubes, centrifuged for solid and liquid phase separation and decanted (removal of cocaine dissolved in methanol). The resulting residue* was washed with ethanol to be finally reunited in 10 ml tubes, until reducing all solid phase in one tube. Thereafter, protoplast content was removed by proceeding acetolysis[7], consisting on an acid hydrolysis through a mixture of acetic anhydride and sulfuric acid (ratio of 9:1). To conclude, the final residue was washed several times in distilled water and microscopical slides were prepared.

To identify pollen grains and spores from cocaine samples, comparisons were conducted using Pollen atlases and modern pollen reference collections, which also contain acetolysed grains.

3. Results and discussion

Cocaine extraction residues that were stained by using Safranin-O resulted in very dark pollen and spore grains, thus hindering observation. For example, the grain depicted in Figure 1A shows no distinct delimitation of its apertural format, structure and surface ornamentation pattern. It becomes clear that the remaining protoplast content hampered the identification procedure. The same difficulty applies to the pollen grain shown on Figure 1B, in which both apertures and sculpturing features could not be well defined.

On the other hand, pollen grains with very conspicuous morphological traits, do not face the same limitations caused by the staining procedure. Examples are the gymnospermous *Podocarpus* pollen, characterized by its noticeable reticulate air-bladders inserted in the pollen grain per-se at, in this case, a 180º angle (Figure 1C), and *Alchornea* (Euphorbiaceae), easily recognized by its opercula (Figure 1D).

In the case of fully acetolysed grains, morphological traits such as apertural format and number, as well as sculpturing features, exine thickness and other traits become clearly discernible. Figure 2A-C illustrates a medium size tricolporate, isopolar, sub-prolate, subtriangular grain with a uniform microreticulate surface pattern, 2.5 µm wall thickness. Also discernible are the long apertures and the elliptic pore shape, lacking an equatorial band.

* Residue here refers to the solid dark organic cocaine-free precipitate containing pollen and spores, whereas the resulting liquid extraction contains only cocaine dissolved in the methanol aliquot.

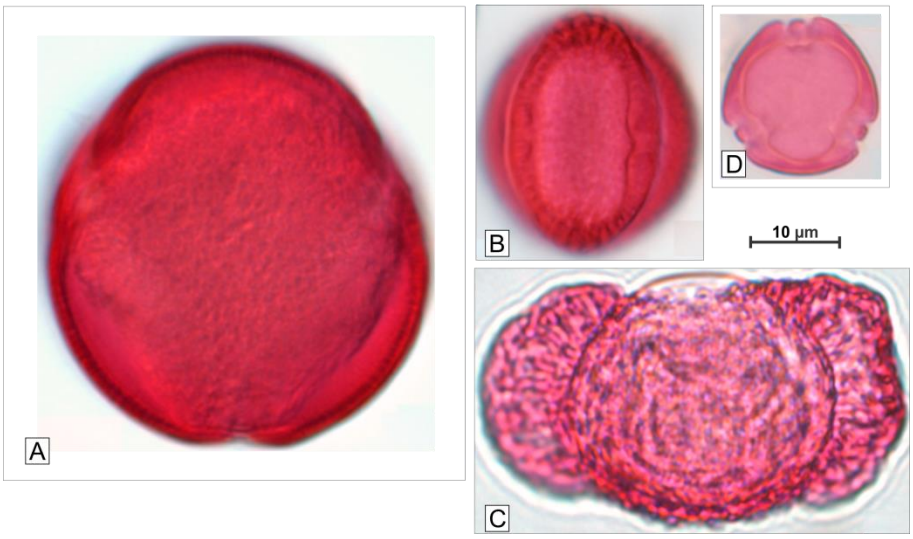

Figure 1. All pollen grains stained, with no Acetolysis. (A) Unidentified pollen grain, polar view. (B) Unidentified tricolporate pollen grain, equatorial view. (C) *Podocarpus*. sp, Podocarpaceae, equatorial view. (D) *Alchornea* sp., Euphorbiaceae, polar view.

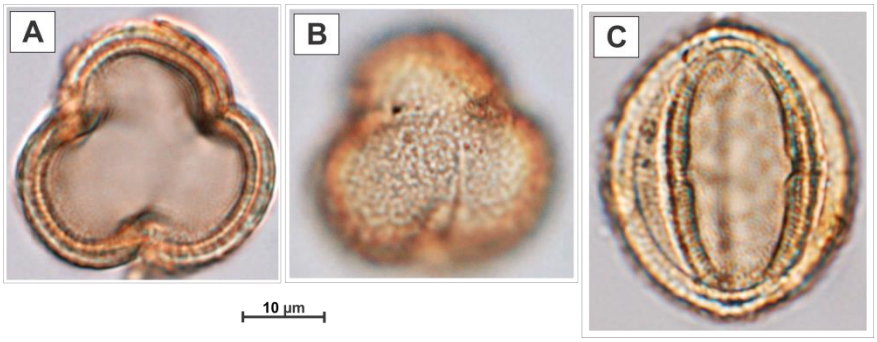

Figure 2. *Sebastiania brasiliensis* pollen acetolised (A-B) polar views. (C) equatorial view.

Thus, with the addition of Acetolysis to this protocol to remove pollen content, we continued recovering pollen grains, despite the inherent methodological loss, and recognized their morphological wall details allowing taxonomic identification. This improvement also enabled measurements which depends on sharp outline view. In our given example from figure 2A-C, a very experienced palynologist is not only able to recognize this pollen grain as belonging to the Euphorbiaceae family, but also to identify it down to genera and species levels.

Analysis of all traits, especially wall thickness measurements, points out to *Sebastiania brasiliensis* Spreng., a small tree common in wetlands and

forests occurring in the southern and southeastern South America, comprising Brazil, Bolivia, Paraguay, Uruguay, Argentina and Chile[12]. *S. brasiliensis* occurs in savannas and in the semi-deciduous forests, a widespread vegetation type in the state of São Paulo, where the cocaine sample was seized.

Considering that tropical regions where coca plants are cultivated are characterized by high rates of biodiversity[13], detailed morphological features are important to distinguish species/genera within the same family. Euphorbiaceae is a well-represented family in South America but pollen genera distinction can enable, at first glance, the exclusion of some large areas covering trafficking routes. Geolocation refinement can be achieved if genera are rarely or narrowly distributed. If species determination is achieved, as in our case, a more precise route can be inferred by this process. Moreover, the larger the number of identified grains, the greater the geolocation precision.

Pollen profile of cocaine samples therefore can enhance the determination of drug routes especially when allied to police reports and chemical analyses performed on the seized drug. In our case sample, if the local law enforcement officials hypothesized that the source was either in Colombia or Peru, the presence of *Sebastiania brasiliensis* would suggest that this sample had been open or repacked in Bolivia and possibly in Brazil. In addition, if we find more pollen grains compatible to the Brazilian Flora, the hypothesis of a local stop would be supported. This is relevant because samples can be reorganized and have their volume redistributed when they travel along the state of São Paulo, throughout clandestine trails, called “countryside routes” [14]. Many of these routes are destined to the city of Santos, where it locates the most important port in South America.

With regard to pollen counts necessary for geolocation reconstruction, this adapted method should be adopted only for large samples, at least 100 grams per sample in order to increase the number of pollen grains in the analysis and the probability to find endemic types. Thirty-three pollen grains and spores (i.e. grains from Lecythidaceae, Fabaceae, Urticaceae/Moraceae, Cyperaceae, Euphorbiaceae, Asteraceae and Loranthaceae families, *Podocarpus*, and trilete as well as monolete spores) were counted from only five slides, which means it not required to use larger cocaine samples for most cases. Consequently, the palynological content of packages, utensils and other

objects related to cocaine handling should be investigated by following different protocols and methodological strategies to compensate for the low number of grains that can be recovered from these materials. We recommend the assessment of pollen and spore concentrations, i.e. number of grains per gram or volume of drug sample, by means of an exotic marker such as *Lycopodium clavatum* tablets[15] traditionally used in ecological studies, thus permitting comparison with other samples by means of different statistical analyses

Obviously, working with higher purity cocaine it is a mandatory condition to indicate sources, once adulterants and diluents may contaminate the sample with allochthonous pollen grains and spores. For this reason, this method is not applicable for low purity cocaine samples.

4. Conclusion

In this contribution, we proposed the use of Acetolysis as one-step procedure added to Stanley's method to recover pollen and spores from cocaine samples, which enhanced visualization of cell wall features, as apertures, ornamentation and layers details, necessary to taxa identification and, consequently, geolocation of cocaine samples.

The innovative forensic practices pointed out in this proposal can contribute to the corroboration or exclusion of investigative lines associated with criminal prosecution, pointing to new technical-scientific means of proof for criminal cases.

Acknowledgments

The authors thank Judge Antonio Maria Patiño Zorz and Superintendence of the Technical-Scientific Police of the State of São Paulo for authorization, Criminal Experts Robson Tirotti Felipe, Elvis Medeiros de Aquino, Fabiane Chaves Cançado, Nicole Tressoldi, Ricardo Ortega, Patricia Hernandez Caleare and Marcelo Henrique Voloch for technical, structural and documental support, and all the staff involved in Drug Seizure of Civil Police and Militar Police from the São Paulo State.

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