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Archaeobotanic: HPLC Molecular Profiles for the Discrimination of Copals in Mesoamerica Application to the Study of Resin Materials from Objects of Aztec Offerings

*Archéobotanique : profils moléculaires par CLHP
pour la discrimination des copals en méso-amérique
Application à l'étude de matières résineuses provenant d'offrandes aztèques*

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Abstract: During archaeological excavations at «Templo Mayor» site, between 2006 and 2008 in Mexico City, thirteen Aztec offerings were found. Among the materials of these offerings the most abundant was a resinous substance used as adhesive, molding material for figures or as a spiritual offering. Two archaeological samples from two Aztec artifacts were analyzed by HPLC. Their global molecular profile was compared to that of fresh botanically certified copal resins from six species of Mexican *Bursera* (Burseraceae family). This study aims to complete a previous research based on GC-MS results that focused only on triterpenic molecular resin composition. The molecular profile of archaeological samples suggests a botanical copal origin related to *B. bipinnata* or maybe *B. stenophylla* species which triterpenic profile composition is identical to.

Résumé : Lors des fouilles archéologiques menées sur le site du « Templo Mayor » entre 2006 et 2008 à Mexico, treize offrandes Aztèques ont été retrouvées. Parmi les objets issus de ces offrandes, le matériau le plus abondant était de type résine sous forme d'adhésif, de matériel de moulage pour des figurines ou encore comme offrande spirituelle. Parmi ces derniers, deux échantillons archéologiques ont été analysés par CLHP. Leur profil moléculaire global a été comparé à celui de résines contemporaines d'origine botanique certifiée provenant de six espèces mexicaines de copal: *Bursera* (famille des Burséracées). Cette étude vient compléter et confirmer les résultats d'une recherche antérieure menée par CPG-SM où seule la fraction triterpénique des résines avait été étudiée. Le profil moléculaire de ces échantillons Aztèques suggère des copals dont l'origine botanique est l'espèce *B. stenophylla* ou bien *B. bipinnata* dont le profil moléculaire triterpénique est identique.

Keywords: archaeobotanic, Aztec, *Bursera*, copal, plant resin, triterpenoids.

Mots clés : archéobotanique, Aztèque, *Bursera*, copal, résine végétale, triterpènes.

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1. INTRODUCTION

The study of archaeological resins can give information relative to the man-environment relationship, the ethnology and technological knowledge of civilizations. In the case of Aztecs, these information are highly valuable because of the lack of documents on pre-Columbian period.

Between 2006 and 2008 archaeological excavations took place at Templo Mayor Site, and a resinous material attracted the attention of archaeologists and conservation professionals, because of their abundant presence in offerings, their versatile use, but also of the diversity on conservation states related to degradation, involving changes in color and surface texture. About offerings context, copal was used as molding material for figurines, spiritual offering and adhesive. To our knowledge formulations related to different uses have never been studied before and the botanical origin of the employed resins is a major concern within archaeobotanic research field.

Historical background and copal in ancient Mexico

The knowledge of art crafts manufacturing in Ancient Mexico is quite ambiguous and most of the time is fragmentary and scarce, due to the lack of documentary sources. This research focuses on the study of two amorphous artifacts, collected from offerings 120 and 126 at Templo Mayor. Table 1 provides additional information about these materials.

The “Templo Mayor” was built on the geographical center of Mexico City. It was a precinct surrounded by a staggered platform, on which the main buildings of the city were located. It was the tributary center of the Empire.

Merchandises from a vast territory either sold in markets or paid as tribute (Carrasco, 1999), were put together in offerings and funerary deposits. In this temple, took place all ritual celebrations: from the regular ones, marked in preColumbian calendar to the most extraordinary ones corresponding to the enthronization and funerary ceremonies for the emperor (Lopez-Luján and Chavez, 2010). In each of these events offerings were buried (Matos-Moctezuma, 2011). When Aztecs arrived to Mexico Valley the Tecpanecs from Azcapotzalco dominated the region. After a short period they established in Chapultepec from which they were expelled. Then they settled in Tizapan a territory dominated by Culhuacan, but they left this settlement because of the hostile environment. The Mexicas or Aztecs were an ethnic group with nahua filiation. After a long peregrination, they founded Mexico-Tenochtitlan, a city that between the XVth and XVIth centuries became the center of one of the biggest empires in Mesoamerica. Aztecs developed one of the richest and more complex religious, political, cosmologic, astronomic, philosophic and artistic traditions.

The offerings found in the Templo Mayor of Tenochtitlan are made by sets of objects carefully ordered in layers (López-Lújan, 1993). Some of these were placed in stone boxes underneath the buildings, whereas others were buried directly in the constructive filling materials between constructive stages of Templo Mayor pyramid. Among the elements gotten back from these offerings were: figurines representing deities, scepters, a pectoral, masks, lap-pets, flutes, whistles, drums, instruments of sacrifice, a wooden mask, flora and fauna remains, etc (López-Lújan and Chavez, 2010). The first Spanish people on American continent kept the Mexican term “copal” as well as the term “incienso de indias” (incense from India) to refer to the natural resins, produced by New World trees, that were

ID	Artifact number	Nature of the material	Weight of sample (g)	Number of Offering	Picture	Scale
A	A 252	Amorphous material	0.04	126		5 mm: 80x
B	Unknown	Amorphous material	0.32	120		5mm: 25x

Table 1: Archaeological samples information

Tableau 1 : Informations sur les échantillons archéologiques

burnt during religious ceremonies, probably because of the parallel use in Europe of *Boswellia* resins (frankincense). Mesoamerican cultures considered copal as a protector. The representation of copal can often be found in codex where priest are offering it to the gods. In goldsmith, copal was used as wax is used in nowadays, in the “lost wax” technique. It was part of the formulas that were used as adhesives for masks, small rocks, and even in dentistry where it was used in dental incrustations, in teeth cavities and in dental restorations as well (Bucio *et al.*, 2006).

Natural plant resins: botanic and chemical composition

To our knowledge few researches deal with the chemical composition of mexican copal. Some articles describe Mexican commercial resins (De-la-Cruz-Cañizares *et al.*, 2005; Orta-Amaro, 2007; Vandenabeele *et al.*, 2007; Hernández-Vázquez *et al.*, 2010;) and it is possible to find papers based on the study of archaeological objects (Magar and Menar, 1995; Stacey and Cartwright, 2006 and Vandenabeele, 2003). They respectively analyzed a turquoise mosaic, a series of objects from the British Museum and a figure molded in resin. Finally a recent study from our research team (Lucero-Gómez, 2012, Lucero-Gómez *et al.*, 2014) focused only on the study of the triterpenic composition of certified origin resins.

Mexican copal resins are produced by trees that belong to the genus *Bursera* spp. from Burseraceae family. Currently, in Mexico, there are more than 80 species of resin producing *Bursera* trees (Rzendowski *et al.*, 2005). *Bursera* species are highly resinous and release a characteristic pine-lemony odor when a leaf or branch is broken. The smell is due to highly volatile terpene compounds such as α -pinene, β -phelandrene, limonene, carene, etc. (Becerra *et al.*, 2001). Oleoresins are referred to as “oilyresins” when they are very fluid. This fluidity is due to a high ratio of volatile to non-volatile terpenes (Langenheim, 2003).

In Burseraceae, triterpenoids are the primary components of the non-volatile fraction.

It is important to note that some other substances can be intermixed or mistaken with resins, among which gums can be mentioned (Langenheim, 2003). Chemically gums are composed of complex chains of hydrophilic polysaccharides derived from monosaccharide molecules such as galactose, arabinose, and rhamnose. Whistler (1993) described in detail the very complex structure of exudated gums.

Given the large number of possible botanical origins for historical samples, the purpose of this research was to establish molecular profiles that allow the discrimination

of botanical origin of samples, so it was necessary to analyze resins from botanically-certified origin. The choice of the botanical species was based on specialized literature and more precisely on previous works about Mexican copal (Magaloni *et al.*, 1993; Stacey and Cartwright, 2006; Montúfar, 2007; Hernández-Vázquez *et al.*, 2010) to increase the possibilities of achieving our goal. Six species were selected for this study: *B. bipinnata*, *B. stenophylla*, *B. simaruba*, *B. excelsa*, *B. laxiflora* and *B. penicillata*.

The difficulty of plant resins analyses relies on the fact that no general analytical technique exists to characterize the molecules entering in their composition and their degradation products (Colombini *et al.*, 2000). The second difficulty in analyzing these materials by HPLC relies on the nature of the resins themselves, which are chemically very complex (Witte *et al.*, 1986). Indeed, *Bursera* resins consist of a wide variety of compounds with very similar structures. This molecular complexity increases with degradation process. Additionally, analysis of archaeological samples is difficult because of the restrictions on the number of samples and their size.

Chemometrical Approach

HPLC-UV/Vis is a reasonably sensitive analytical tool that allows molecular profiling. In this research, this technique was used coupled to two multistatistical methods PCA (Principal Component Analysis) and LDA (Linear Discriminant Analysis).

A number of papers have identified natural materials by means of chemometric approaches (Tapp *et al.*, 2003; Rezzi *et al.*, 2005; Armanino *et al.*, 2008; Casale *et al.*, 2010a, Casale *et al.*, 2010b). The most common is PCA (Principal Component Analysis), which has been applied to the discrimination of proteinaceous media (Colombini *et al.*, 1999; Andreotti *et al.*, 2006; Bonaduce *et al.*, 2006; Gautier and Colombini, 2007), drying oils (Andreotti *et al.*, 2006; Colombini *et al.*, 1999), polysaccharides (Colombini *et al.*, 2002) and resins (Vieillescazes *et al.*, 1996).

In the present work, this approach allowed a better discrimination of chromatographic profiles in order to enhance interpretation of analytical results. A description of the statistical tools employed can be found in the methods section of this work.

2. MATERIAL AND METHODS

A protocol using HPLC (High Performance Liquid Chromatography) was employed in this research.

Chromatographic techniques are powerful tools applied in several laboratories specialized in artworks because of their ability to separate organic compounds from complex mixtures (Doménech-Carbó, 2008).

Statistical Methods:

Principal Component Analysis (PCA)

The aim of this method is to capture the intrinsic variability in the data and at the same time, to reduce the dimensionality of a data set, either to ease interpretation or as a way to avoid overfitting and to prepare for further analysis (Terradez, 2000). In this way PCA succeeds in explaining the maximum amount of variance with the fewest number of main components (the smallest loss of information). Principal components are linear combinations of the original variables with the first principal component having the largest variance, the second having the second largest variance and so on (Cosio *et al.*, 2007). PCA also allows considering the preferred directions of the data and ignoring the variables that do not have a big influence in the separation of the groups (Barreto, 2009). When data is projected in two dimensions (or three), it is possible to distinguish visually the different classes that could appear, grouped in the projection. The different classes will be formed by those observations that display an uniform behaviour to each other and differ from other existing groups (García, 2002).

Statistical Methods:

Linear discriminant analysis (LDA)

LDA is a powerful chemometric tool that allows finding a predictive classification model, meaning that it allows the construction of a model able to foretell the property of a sample according to a previously defined category, or to investigate the way variables contribute to the separation in categories. This means that it allows studying the degree in which different populations, established *a priori*, differ from each other. It deals with two kinds of problems: descriptive discrimination, in which it describes how two (or more) populations differ from each other; and classification in strict sense, in which there are two or more given populations and an object that can belong to one of them. The discriminating analysis requires to obtain a linear (or nonlinear) combinations of independent variables that will discriminate between defined groups *a priori*, so that the errors of the wrong classification must be minimum (Barreto, 2009).

The LDA model is based on a set of samples of well-known category, called training data set, and then a regres-

sion equation is used as discriminating function (Casale *et al.*, 2010a).

Once the model is obtained, it is used for the prediction of the category of property of the samples of unknown category. LDA can be linear or quadratic. The linear model assumes that all the groups have the same covariance matrix, while quadratic ones do not make this assumption.

In this research a level-one-out procedure was used. This means that each sample was once removed from the data set. The classification model is rebuilt and the removed sample is classified in this new model. All the samples of the data set are sequentially removed and reclassified. Finally a percentage of the correct classification is calculated (Lachlan, 1992). The quality of the LDA classification model was modeled according to the basis of the validation results. The statistic analysis (PCA and LDA) in this work was performed using Minitab 16 (Minitab Inc. PA, California).

Reagents

Standard molecules were used for their identification in molecular composition of archaeological resins: α -amyrin, β -amyrin, lupeol, and lupenone were purchased from Extrasynthèse (Genay, France), while α -amyrone and β -amyrone were purchased from BCP instruments (Irigny, France), 3-*epi*-lupeol, 3-*epi*- α -amyrin, 3-*epi*- β -amyrin were isolated by our team from commercial resin samples (Culioli *et al.*, 2003).

Samples of resin from botanical certified origin

Certified resin samples from six species of *Bursera* trees were collected in Mexican territory. The choice of these particular species was defined by two factors: previous research results (Stacey and Cartwright 2006; Montúfar, 2007 and Hernández-Vázquez 2010) and to obtain an overview on the variability of the chemical composition of *Bursera* resins from Mexico. Moreover, to rule out different molecular compositions of resin from the same species, samples were taken from different trees.

For the collection of the certified-origin samples, this research was realized with the invaluable collaboration of experts in botanic (Dr. Rito Vega) and in biomaterials (Dr. Irma Belio) from the Autonomous University of Sinaloa (UAS), as well as with the cooperation of the academic team of Biomaterials from the UAS (UAS CA-208). The collection was realized from summer to fall, period during trees have both leaves and fruits, which are necessary for botanical identification. After practicing a small cut into tree barks, resin was collected in glass tubes. In table 2, information

relating the number of samples for each species and their geographical location (GPS position) are presented.

Table 2]

Historical samples

The two archaeological samples come from offerings discovered at Templo Mayor Site. Sample referred “A” comes from offering 120 while sample “B” comes from offering 126, both offerings were unearthed in a lot where once stood a colonial building known as “Casa de las Ajaracas”. The principal stairs of the sixth constructive stage of the Templo Mayor were built in the same physical space that “Casa de las Ajaracas” (Matos, 2007).

Gum analysis

The aim of these analyses is to evaluate the gum percentage in the raw resinous material. The gum part is hydrosoluble and the resinous part is soluble in methanol. This study was performed solving a known amount of fresh resin in methanol. After sonication and centrifugation, the residue left in the bottom of the solution was dried and weighed. The percentage of gum was calculated from this data. Finally, water was added to ensure the polarity of the residue and to verify its total solubility.

Analytical conditions in HPLC-UV/Vis

For the HPLC study, both standards and global resins were solved in methanol (analytical grade, Merck). The protocol consists in solubilizing an amount of crude resin (variable depending on botanical origin) in 2 mL of this solvent, then the solution was sonicated during 10 min. After centrifugation, the supernatant was injected into the HPLC system. Each sample was injected in triplicate to ensure reproducibility of the results.

Analyses were performed using a reverse phase column with an elution gradient. The type of column used in this research field is essential, taking into account the previous results obtained by our team. Indeed, these works (Mathe *et al.*, 2004), showed that for resins, a column in reverse phase C18 led to satisfactory separations and resolutions of the compounds. This study was thus carried out using a column with non-polar stationary phase Merck Lichrocart Purostar 5 μ m 100 RP-18e 250 mm x 4 mm (Merck, Darmstadt, Germany).

LC-PDA analysis was carried out in a Waters liquid chromatograph consisting of a high-pressure ternary pump Waters 600, a vacuum degasser, a high pressure manual injection valve (20 μ L injection loop) and a photodiode array detection (PDA) system Waters 2996. The system was controlled by Empower 2 software. The LC separation was performed at 35° C with a mobile phase consisting of a binary elution composed of (A) bidistilled water containing 0.01% trifluoroacetic acid (TFA) and (B) methanol analytical grade (Merck). The chromatograms were acquired at 210 nm. Identification of original triterpenoids was based on the co-injection of samples with reference molecules. Chromatographic analyses of standard molecules, certified resins and archaeological sample A were carried out for 35 min at a continuous flow-rate of 1 mL/min. For the study of archeological sample B, a prolonged gradient was necessary as compounds that were not present in fresh samples were detected. The gradient profile is presented in table 3.

3. RESULTS OF HPLC-UV/Vis STUDY

Standard molecules

Liquid chromatography was applied to the study of six standard molecules (table 4) at 210 nm, which is the general wavelength number for the study of resins (Büchle *et al.*, 2003; Mathe *et al.*, 2004).

Botanical species	Number of collected samples	Date of collection (dd/mm/yyyy)	GPS localization	
<i>B. bipinnata</i>	11	09/08/2010	25°07'21.42'N	107°11'57.42'W
<i>B. excelsa</i>	4	05/09/2009	24°50'37.62'N	107°02'28.76'W
<i>B. laxiflora</i>	14	01/11/2009	24°45'36.06'N	107°11'51.48'W
<i>B. penicillata</i>	6	29/08/2009	24°46'08.64'N	107°09'28.26'W
<i>B. simaruba</i>	15	01/11/2009	24°51'27.60'N	107°01'07.02'W
<i>B. stenophylla</i>	8	19/09/2009	26°41'26.40'N	108°19'58.70'W

Table 2: Number of certified samples studied and their geographical location

Tableau 2 : Nombre d'échantillons certifiés étudiés et leur emplacement géographique

Time (min)	0	10	15	35 ^a	45 ^b
% Methanol	85	95	100	100	100
% H ₂ O-TFA	15	5	0	0	0
Flow : 1 mL.min ⁻¹		Temperature of the column = 35 °C			

Table 3: HPLC gradient profil (^a Final run time for standard molecules, certified resins and archaeological sample A; ^b Final run time for sample B).

Tableau 3 : Gradient CLHP (^a Temps final d'analyse pour les molécules standards, les résines certifiées et l'échantillon archéologique A; ^b Temps final d'analyse pour l'échantillon archéologique B).

Their retention time under the designed gradient is shown in table 4. These triterpenes were assigned by a number, according to their elution order. Number assignment will be kept all along this paper for the identification of these molecules in chromatograms.

Results of HPLC-UV/Vis study on certified resins samples

The first difficulty for the study of resins by HPLC-UV/Vis arose because of the need of different amounts of sample depending on the apolar molecules content of the studied species: going from 2 mg up to 30 mg, as referred to in table 5.

The amount of resin needed for the analysis has been directly correlated to the amount of gum present in the sample: the higher amount of gum was, the rarer apolar compounds were (terpenoids included) and so, the more important the sample amount needed for the analysis

Table 5 also sums up the percentage of gum found in each studied species. Generally gum ratio is correlated to the producing species but this fact alone cannot constitute a factor for botanical identification, as gums are water soluble, widely distributed, and easy degradable materials. The important proportion of gum in *B. simaruba* exudates provided chromatograms with less resolved peaks than in other species, therefore the results of analysis of this species could not be treated by PCA and LDA statistical analysis.

Chromatograms in liquid phase allowed a very clear interspecies distinction. It is note-worthy that two separated areas were distinguished in each chromatogram: the first one going from 0 to 24 minutes and the second running from 24 to 35 minutes. The first zone corresponds to components with lower molecular weight and highest polarity. This first molecular population was characterized by a succinct LC-SM analysis as sesquiterpenes, however these peaks cannot be surely assigned in the context of this article. The second group of peaks in the chromatogram corresponds to molecules of higher molecular weight and lower polarity; nine of the eleven molecules detected in this

N°	Molecule	Retention time (min)
1	3- <i>epi</i> -lupeol	26.5
2	lupeol	27.6
3	3- <i>epi</i> -β-amyryn	28.2
4	lupenone	28.7
5	3- <i>epi</i> -α-amyryn	29.4
6	β-amyryn	29.9
7	α-amyryn	31.3
8	β-amyrone	31.4
9	α-amyrone	32.2

Table 4: Retention time of standard molecules

Tableau 4 : Temps de rétention des molécules standards

	Mass of sample used for HPLC analysis	% Gum	% Resin
<i>B. bipinnata</i>	3 mg	0	100
<i>B. excelsa</i>	6 mg	0.5	99.5
<i>B. simaruba</i>	30 mg	25	75
<i>B. penicillata</i>	3 mg	0	100
<i>B. stenophylla</i>	3 mg	0	100
<i>B. laxiflora</i>	6 mg	8	92

Table 5: Amount of resins used for the HPLC chromatographic study and gum-resin percentage in resin samples from certified species.

Tableau 5 : Quantité de résines employée pour l'analyse chromatographique et pourcentage en gomme-résine des résines certifiées botaniquement.

region were identified by co-injection with standard molecules as triterpenes.

Figure 1 shows a chromatogram of *B. bipinnata* where the two above mentioned areas of peaks are distinguished: marked with a continuous arrow the more polar zone, and with a dotted one the triterpenoid zone. For the first part of the chromatogram we chose a notation for compounds beginning either with letters A or B.

Compounds noted "A" are widely distributed in the six species, most of the time A1 is a major peak in the chromatograms. These peaks were present also in chromatograms of

Figure 1: Chromatogram of *B. bipinnata* (sample 11d2), triterpenoid zone running from 24 to 35 min in retention time. (1) 3-*epi*-lupeol, (2) lupeol, (3) 3-*epi*- β amyrin, (4) lupenone, (5) 3-*epi*- α amyrin, (6) β -amyrin, (7/8) α -amyrin/ β -amyrone, (9) α -amyrone).

Figure 1 : Chromatogramme de *B. bipinnata* (échantillon 11d2), agrandissement de la zone triterpénique de 24 à 35 min. (1) 3-épi-lupéol, (2) lupéol, (3) 3-épi- β amyrine, (4) lupénone, (5) 3-épi- α amyrine, (6) β -amyrine, (7/8) α -amyrine/ β -amyrone, (9) α -amyrone).

archaeological samples. While “B” compounds are specific for each species, meaning that their presence and quantity varies a lot among studied species and were almost absent from archaeological samples.

Table 6 resumes the presence and absence of compounds of lower molecular weight and higher polarity, and table 7 sums up the presence and absence of triterpenoids related to botanical origin.

Concerning resolution of peaks, in our analytical conditions, it was impossible to separate the signals from α -amyrin (7) and β -amyrone (8), which is not surprising as both compounds have a similar molecular structure.

The closest triterpenic molecular composition was found between *B. bipinnata* and *B. stenophylla* species. A difference between *B. bipinnata* and *B. stenophylla*, lies in the absence of triterpenic compounds T1 at 25.4 min and T2 at 33.5 min in *B. bipinnata* (Table 7, Figure 2). Gum was absent of the composition of both species.

Concerning *B. excelsa* it was the species that displayed the poorest composition in the triterpenic zone, only four compounds were detected by HPLC-UV/Vis: lupeol (2), 3-*epi*-lupeol (3), lupenone (4) and β -amyrin (6). Nevertheless relative peak areas of β -amyrin (6) and lupenone (4) were considerably smaller than the ones for those of the other two

ID of the peak	A4	A3	A2	A1	B1	B2	B3	B4	B5	B6	B7	B8
Ret. time (min)	8.9	10	11.4	12	16	16.5	17	18	19.4	20	20.6	24
<i>B. bipinnata</i>	-	+	+	+	-	-	-	+	+	-	+	-
<i>B. stenophylla</i>	+	+	+	+	-	-	-	+	+	-	+	+
<i>B. excelsa</i>	-	+	-	+	+	-	-	-	+	+	-	+
<i>B. laxiflora</i> (profile A)	+	+	+	+	+	+	-	-	-	-	-	+
<i>B. laxiflora</i> (profile B)	-	-	+	+	+	+	-	-	-	-	-	+
<i>B. penicillata</i>	-	-	-	+	+	-	+	+	+	-	-	-
<i>B. simaruba</i>	-	+	+	+	-	-	-	-	-	+	-	-
Archaeological sample A	-	+	-	+	-	-	-	+	-	-	+	+
Archaeological sample B	+	-	-	+	-	-	-	+	-	-	-	-

Table 6: Retention time of peak corresponding to non triterpenic compounds observed in each species (+ presence, - absence)

Tableau 6 : Temps de rétention des pics correspondants aux composés non triterpéniques de chaque résine (+ présence, - absence).

ID of the peak	T1	3- <i>epi</i> -lupeol	lupeol	3- <i>epi</i> - β -amyrin	lupenone	3- <i>epi</i> - α -amyrin	β -amyrin	α -amyrin/ β -amyrone	α -amyrone	T2
Ret. time (min)	25.5	26.5	27.6	28.2	28.7	29.4	29.9	31.3	32.1	33.5
<i>B. bipinnata</i>	-	+	+	+	+	+	+	+	+	-
<i>B. stenophylla</i>	+	+	+	+	+	+	+	+	+	+
<i>B. excelsa</i>	+	+	+	-	+	-	+	-	-	-
<i>B. laxiflora</i> (profile A)	-	+	+	+	+	-	+	+	+	-
<i>B. laxiflora</i> (profile B)	-	+	+	-	+	+	+	+	+	-
<i>B. penicillata</i>	-	+	+	+	+	+	+	+	+	-
<i>B. simaruba</i>	-	+	+	+	+	+	+	+	+	-
Archaeological sample A	-	+	+	-	+	+	+	+	+	+
Archaeological sample B	-	+	+	-	+	+	+	+	+	+

Table 7: Retention time of peaks corresponding to triterpenic compounds (+ presence, - absence).

Tableau 7 : Temps de rétention des pics des composés triterpéniques (+ présence, - absence).

A) *B. bipinnata* **B) *B. stenophylla*** Figure 2- Comparison of A) *B. bipinnata* and B) *B. stenophylla* chromatograms. (1) 3-*epi*-lupeol, (2) lupeol, (3) 3-*epi*- β -amyrin, (4) lupenone, (5) 3-*epi*- α amyrin, (6) β -amyrin, (7/8) α -amyrin/ β -amyrone, (9) α -amyrone.Figure 2- Comparaison des chromatogrammes de A) *B. bipinnata* et B) *B. stenophylla*. (1) 3-*épi*-lupéol, (2) lupéol, (3) 3-*épi*- β -amyrine, (4) lupénone, (5) 3-*épi*- α amyrine, (6) β -amyrine, (7/8) α -amyrine/ β -amyrone, (9) α -amyrone.

compounds, and in the apolar zone A1 was the major peak. On the other hand gum content of samples of this resin reaches almost zero (0.5%).

B. simaruba resin exhibited the higher content of gum (25%). The presence of gum complicated HPLC analysis: resolution of peaks corresponding to triterpenes in its chromatograms was poorer, three clumps of peaks were observed, the first one consisting of 3-*epi*-lupeol, lupeol, 3-*epi*- β -amyrin and lupenone while the second one 3-*epi*- α amyrin and β -amyrin, the third included the peak comprising 7/8 α -amyrin/ β -amyrone like the other species plus α -amyrone, and in the more polar zone of the chromatogram A1 was outnumbering the other peaks.

B. penicillata exhibited a high number of peaks in the first zone of the chromatogram, where A1 was the highest, other peaks with high relative area were B2, B3 and B5. Concerning the triterpenic zone, nine standard molecules were identified; the only triterpenes absent from its composition were T1 and T2.

Finally *B. laxiflora* resins were the ones that displayed the greater diversity in molecular composition in this study. Among the samples analyzed, two lots could be distinguished, given their diverse molecular profile. Nevertheless some common features could be distinguished between the two lots: B1 had the peak with the biggest area in all the cases. Concerning triterpenic fraction, the relative peak area of triterpenic compounds was rather small. In "lot a", triterpenic most important peaks were those from 3-*epi*-lupeol, lupeol and 3-*epi*- β -amyrin, while in "lot b" the main signal in the triterpenic zone was 3-*epi*-lupeol.

Results of HPLC-UV/Vis study on archaeological resins

In archaeological samples, the percentage of gum was null: this fact can be correlated either to the botanical origin of the sample or to the fact that these samples were exposed during a long time to high moisture and even under-water conditions. Nevertheless sample B contained a small proportion (1.73) of matter that was not soluble in our experimental conditions. The small amount of this material available for analysis prevented further studies on its nature.

Despite the age of archaeological samples and their preservation under different conditions of relative humidity, pH and temperature, their molecular composition (appearing in chromatograms) retained most of the triterpenic fraction compounds except for 3-*epi*- β -amyrin and T1, as it has been said above. Within the more polar fraction the most important signal arose from compound A1.

The exceptional conservation state of these samples can be attributed to burial anoxic environment with a high humidity level, the absence of light, and more or less stable temperature.

HPLC chromatogram profiles from Aztec materials coming from Templo Mayor may suggest that they are either from *B. bipinnata* or *B. stenophylla*. In order to enhance the exploitation of these results, and to find a more concrete answer regarding the botanical origin of these samples, two statistical methods were used to analyze the data.

Concerning the molecular profile, the archaeological samples studied were different: in sample B four molecules that were not present in fresh samples were detected, this was the reason that made the use of a longer gradient necessary. These compounds were absent from sample A.

In figure 3, the chromatogram corresponding to sample B is displayed; the peaks can be appreciated from 32.5 to 36 min identified as C1 to C3. These compounds may correspond to apolar molecules. In figure 4, the chromatogram of sample A is presented.

From these chromatograms, important informations can be extracted concerning ageing: some molecules disappeared in some cases like A2, A3, B8 Molecules that were totally absent from these samples and that therefore are more prone to degradation are: B1, B2, B3 and B5.

Finally sometimes molecules related to ageing like C1 to C3 may appear. However some compounds resist to ageing like triterpenes (1-8).

HPLC coupled to PCA analysis for botanical certified resins

For this study, both polar (table 6) and triterpenic compounds (table 7) were taken into account. Peak areas from chromatograms were used in an indirect way: as HPLC analyses were performed on different amounts of crude resin, we chose to use the relative area percentage of each peak for PCA and LDA analysis.

B. simaruba was excluded from statistical analysis because of the poor resolution achieved that prevented distinction of the peaks of triterpenic compounds. A data matrix with 40 rows (resin of certified origin samples) and 22 columns (variables, in this case relative area percentage of the assigned peaks in the chromatogram) was built. Figure 5 shows the graphic results of PCA. The structure of the data, using the first two principal components is displayed.

In this case the first two principal components were enough to display the data structure, and they explained 46.5% of the total variance. By examining the score plot in the area defined by the first two principal components,

Figure 3: Chromatogram of archeological sample B with triterpenes and ageing related products (C1-C3). 1) 3-*epi*-lupeol, 2) lupeol, 3) 3-*epi*- β -amyrin, 4) lupenone, 5) 3-*epi*- α amyrin, 6) β -amyrin, 7/8 α -amyrin/ β -amyrone.

Figure 3 : Chromatogramme de l'échantillon archéologique B avec la présence de triterpènes et de composés de dégradation (C1-C3). 1) 3-*épi*-lupeol, 2) lupéol, 3) 3-*épi*- β -amyrine, 4) lupénone, 5) 3-*épi*- α amyrine, 6) β -amyrine, 7/8 α -amyrine/ β -amyrone.

Figure 4: Chromatogram from archeological sample A. 1) 3-*epi*-lupeol, 2) lupeol, 3) 3-*epi*- β -amyrin, 4) lupenone, 5) 3-*epi*- α amyrin, 6) β -amyrin, 7/8 α -amyrin/ β -amyrone, 9) α -amyrone.

Figure 4 : Chromatogramme de l'échantillon archéologique A. 1) 3-*épi*-lupeol, 2) lupéol, 3) 3-*épi*- β -amyrin, 4) lupénone, 5) 3-*épi*- α amyrine, 6) β -amyrine, 7/8 α -amyrine/ β -amyrone, 9) α -amyrone.

good discrimination results were achieved, furthermore even *B. bipinnata* and *B. stenophylla* were correctly discriminated. *B. penicillata* is also well separated from the other species. Concerning *B. excelsa* and *B. laxiflora* they were grouped into the same area of hyperspace. Both species have a lower content of triterpenes compared to the other studied species, and this factor leads to a loss of sensibility in their discrimination using PCA with HPLC data (Figure 5).

When analyzing the loading plot (Figure 6), the molecules that exhibit the highest influence onto first component with positive values are peaks corresponding to triterpenes: lupeone, 3-*epi*- α -amyrin, lupenone, α -amyrin/ β -amyrone. On the negative side with the highest impact B8 and B1 from the more polar fraction have a highest discrimination power.

On the negative part of first component B2, B5, A3 and A4, all volatile compounds have been shown to have a higher impact, together with 3-*epi*-lupeol from triterpenic fraction.

On the second component B3 and B5 have the higher impact in discrimination, they both seem to own the same information so for further analysis B5 may be taken out of the analysis as it exhibits a slightly lower impact on both components. Along with B3 and B5, α -amyrone was found to have a high impact on second component. On the negative part of the second component A3, A4 and 3-*epi*-lupeol display the higher discrimination power.

Generally all triterpenes play a major role into discrimination except for lupeol which seems to have little impact on both components, and A1 which also exhibits a very

Figure 5: Distribution PCA of the resins samples from botanical certified origin with HPLC data. ● *B. laxiflora*, ◆ *B. excelsa*, ■ *B. stenophylla*, ▲ *B. bipinnata*, ▼ *B. penicillata*.

Figure 5 : Représentation ACP des résines certifiées botaniquement, en les données CLHP. ● *B. laxiflora*, ◆ *B. excelsa*, ■ *B. stenophylla*, ▲ *B. bipinnata*, ▼ *B. penicillata*.

Figure 6: Loading plot for PCA analysis using HPLC data for botanical certified resins.

Figure 6 : Graphique des variables en ACP utilisant les données HPLC des résines certifiées botaniquement.

limited influence into discrimination. This is reasonable if its ubiquity is considered.

Linear Discriminant Analysis

A classification model was built based on the same matrix used for PCA. LDA analysis was applied in order to find a predictive classification model, able to separate the five botanical species. Results for fitting analysis are shown on table 8 and in table 9 for validation matrix.

The confusion matrix (table 9) allowed a global recognition percentage of 95.0% while only one sample of *B. excelsa* and one from *B. stenophylla* samples were incorrectly classified in the validation procedure.

This can be considered as a consequence of dealing with classification from a sample of *B. stenophylla*, so that their probability to be correctly classified by this model is 87.5%. On the other side *B. bipinnata* was always correctly classified. When looking at figure 5 with PCA classification, a greater dispersion of the individuals corresponding to *B. stenophylla* can be noticed, this fact leading to a greater possibility of misclassification of resin from this origin, when applying this model with the present data.

HPLC coupled to PCA analysis for archaeological resins

Data concerning the two archaeological samples from Templo Mayor were added into the matrix in an attempt to achieve the discrimination of their botanical origin. In this graph, it could only confirm the previous interpretation based only on chromatograms in which an origin between *B. bipinnata* and *B. stenophylla* is suggested (figure 4). Given the closeness of these two species in the hyperspace it was not possible to assess with certainty the botanical origin of the archaeological samples. This fact is also connected to the molecular changes that take place during ageing processes.

4. CONCLUSION – DISCUSSION

The usefulness of HPLC-UV/Vis was undeniable concerning molecular profiling for differentiation between resins with different botanical origin. Currently the botanical distinction between *B. stenophylla* and *B. bipinnata* is unclear and the use of very techniques like HPLC-UV/Vis allows obtaining slight differences in the molecular composition of their resins. This closeness on molecular composition confirms the genetic closeness of these two species.

The advantage of coupling this liquid chromatographic technique with statistical methods such as PCA and LDA was to obtain a model able to discriminate botanical origin of fresh resins from species with similar molecular compositions such as *B. bipinnata* and *B. stenophylla*, which were difficult to differentiate using only chromatographic results. The performance of the mathematical model constructed with HPLC data in LDA shows a high result with 100% recognition in the fitting matrix and a 95% of positive recognition in the cross validation matrix.

Put into group	<i>B. bipinnata</i>	<i>B. excelsa</i>	<i>B. laxiflora</i>	<i>B. penicillata</i>	<i>B. stenophylla</i>
<i>B. bipinnata</i>	10	0	0	0	0
<i>B. excelsa</i>	0	4	0	0	0
<i>B. laxiflora</i>	0	0	12	0	0
<i>B. penicillata</i>	0	0	0	6	0
<i>B. stenophylla</i>	0	0	0	0	8
Total N	10	4	12	6	8
N correct	10	4	12	6	8
Proportion	1	1	1	1	1
N = 40		N Correct = 40		Correct Proportion = 100%	

Table 8: Fitting matrix of the LDA Rows represent the true class, and the columns the assigned class.

Tableau 8 : Matrices d'ajustage en analyse linéaire discriminante, les lignes représentent la vraie classe d'appartenance, et les colonnes les classes attribuées.

Put into group	<i>B. bipinnata</i>	<i>B. excelsa</i>	<i>B. laxiflora</i>	<i>B. penicillata</i>	<i>B. stenophylla</i>
<i>B. bipinnata</i>	10	0	0	0	1*
<i>B. excelsa</i>	0	3	0	0	0
<i>B. laxiflora</i>	0	1*	12	0	0
<i>B. penicillata</i>	0	0	0	6	0
<i>B. stenophylla</i>	0	0	0	0	7
Total N	10	4	12	6	8
N correct	10	3	12	6	7
Proportion	1	0.75	1	1	0.875
N = 40		N Correct = 38		Correct Proportion = 95%	

Table 9: Confusion matrix of LDA, rows represent the true class, and the columns the assigned class. Wrong assignments are marked with a *.

Tableau 9 : Matrices de confusion en ADL, les lignes représentent la vraie classe et les colonnes la classe attribuée. Les attributions incorrectes sont marqués d'une *.

Figure 7. Distribution PCA of the resins samples from botanical certified origin including archaeological samples with HPLC data. ● *B. laxiflora*, ◆ *B. excelsa*, ■ *B. stenophylla*, ▲ *B. bipinnata*, ▼ *B. penicillata*, ● A, ● B.Figure 7. Représentation ACP des résines certifiées botaniquement et des échantillons archéologiques. ● *B. laxiflora*, ◆ *B. excelsa*, ■ *B. stenophylla*, ▲ *B. bipinnata*, ▼ *B. penicillata*, ● A, ● B.

Concerning ageing, liquid chromatography confirms that *Bursera* resins undergo significant changes over time: on the one hand a decrease of the polar compounds composition and on the other hand an oxidation and maybe a polymerization of the triterpenic fraction. These modifications can be assessed only partially by means of HPLC-UV/Vis, as it was presented on the chromatograms from archaeological samples and therefore a deepened study should imply the isolation of compounds C1 to C3 and its formal study by N.M.R. or MS-MS. Therefore there is a problem of loss concerning some compounds and the polymerization of others that lead to sometimes drastic changes in proportions between the compounds. It is also thought out to make an artificial ageing study of contemporary resins.

It is interesting to notice that some compounds are not always present in archaeological resins. Therefore an insight into the mechanism of their formation and maybe a correlation between ageing conditions and their presence should be established.

In order to achieve a better botanical discrimination in aged samples, further studies need to be conducted about degradation processes, including the identification of non-triterpenic molecules and also molecules arisen from ageing.

In a second step, the establishment of correlations between products of degradation and stages of ageing could be established. Only a few molecules seem to be stable during natural ageing: A1 of the non-triterpenic fraction and all the triterpenic molecules except 3-*epi*- β -amyrin, which nevertheless exhibited a tendency to proportion change.

Molecular profile of both archaeological resins is quite close despite a slight difference in conservation state. Generally speaking, burial environment contributes to a extent to the good preservation of these materials based on molecular profile for both samples analyzed in this work, even though they were collected from different offerings (offerings 120 and 126). The analysis results on archaeological samples suggest that they could be resins of either *B. bipinnata* or *B. stenophylla* species. Pollen of *B. bipinnata* has been founded by paleobotanists, in archaeological context in the site of Templo Mayor (Montufar, 2007). However, *B. bipinnata* is a species with a wide geographical presence and sometimes it produces hybrids with other members of the *Bullockia* botanical section.

On the historical part, the results of these experiments confirmed the accuracy of information contained in chronicles from 16th century from B. Sahagún about the numerous uses that Aztecs and original people from Mesoamerica gave to copal. This research also yielded important information for archaeologist that was previously unavailable: firstly this work confirms, on the molecular level, that materials found at Templo Mayor offerings are natural solid resins, and that one botanical origin of copal among the great variety of over 80 possible species of *Bursera* available at Aztec empire, seems to be preferred by the priests.

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