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Abstract

Despite the historical richness of ancient records mentioning or describing Chinese ink since the first centuries CE, the manufacture and use of Chinese inks in early history remain obscure. The characterization of inks from dated ancient manuscripts could not only give us new information on the evolution of the manufacture of inks but also on their use.

The purpose of this work was first to optimize and then validate a Pyrolysis-comprehensive two-dimensional gas chromatography/mass spectrometry (Py-GCxGC/MS) approach to identify compounds characteristic of ingredients used in the manufacture of contemporary traditional Chinese inksticks. Performance assessment was accomplished by studying the distribution of compounds characteristic of soot and proteinaceous binder and by performing repeatability tests remaining constant from one analysis to the other.

A strategic sampling protocol and the optimized Py-GCxGC/MS method was then applied for the first time to characterize ancient inks manufactures from tiny fragments of Tocharian manuscripts (6^{th} -8^{th} centuries CE) of the Pelliot Collection stored in the *Bibliothèque nationale de France* as

part of the “History of the Tocharian Texts of the Pelliot Collection” (HisTochText) ERC project. The approach allowed us to distinguish compounds attributable to the ink present from those imputable to the paper support and its preparation. Compounds characteristic of soot as well as of proteinaceous material were identified. Moreover, our data provides conclusive evidence for the use of a wide range of additives in the manufacture of the inks present. Based on the analytical data, this work demonstrates the potential of a Py-GCxGC/MS approach in the characterization of inks from tiny fragments of ancient manuscripts of historical value, making this approach highly relevant to the study of inks manufacture.

Keywords: Chinese inks; Py-GCxGC/MS; Pyrolysis; soot, animal glue, Tocharian manuscripts

1. Introduction

Chinese have discovered the secret of ink since at least the 3rd century BCE, according to archaeological discoveries - such as an ink stone dating back to the Han Dynasty (202 BCE–220 CE) [1] - and ancient texts - such as the poem by Ts'ao Chih (192-224 CE) and its opening line “Ink is derived from the soot of black pine” [2]. However, the first extant description comes from the 5th century CE [3] and the recipe written by Jia Sixie (賈思勰), Northern Wei Dynasty official and author of the *Qimin yaoshu* (齊民要術) a treatise on agriculture [4,5]. Since that time, the Chinese literature on ink manufacture and its technicalities has become extensive and can be accessible to Western readers thanks to diverse translations [6]. They mention a large range of recipes used through time, but all the main ingredients used in manufacturing ink are to be found in this early recipe: a pigment (soot), a binding agent (glue), and miscellaneous additives (chin tree bark, egg whites, cinnabar, musk, etc.).

The soot used in inkstick-making was initially supplied by the pine. Pine branches rich in resin were burnt in ovens and its soot collected. In addition to pine, other types of wood were occasionally used, such as the tree of life (*Thuya orientalis*) and birch wood. However, pine was and remained the classic supplier of wood for the production of soot [1,4,6]. However, during the Song Dynasty (960 – 1279), in view of the increasingly noticeable shortage of wood (at least in the North of China) increasing with the popularity of ink [6], it was only natural to look for other sources of soot. Chinese came up with the idea of burning oil in order to produce ink with it. From the Song Dynasty and onwards, lampblack made from combustion in lamps of animal, vegetable and mineral oils, such as hemp oil, rivalled the use of pine soot [4,6]. Around 1400 CE, soot extraction from pine trees was no longer practiced. Pine soot and oil soot could be also mixed, as well as oil soot and pine resin [6].

The use of soot necessitates the use of a binding agent to hold the particle together in the solid form as well as bind the carbon pigment to the writing surface. The binding agents used in Chinese ink were traditionally glues made from a variety of animal parts (hides, muscles, bones, shells, horns, fish skins, etc.) such as cowhide glue, fish glue, ox horn [4,7].

Since glue sometimes has a strong, unpleasant odor of its own, efforts were made to compensate for this by adding fragrances, but also strengthening the coloring of the ink and for consistency purpose.

Camphor was known as a perfume in the 10th century CE and is still used today in ink making, as well as cloves. The following are also described as additives in the 10th century: honey locus (*Gleditsia triacanthos)* pods, pomegranate skin, copper vitriol, rattan yellow (a dye obtained from *Calamus draco*), croton beans (*Croton tiglium*), safflower (*Carthamus tinctorius*), verbena (*Verbena officinalis L.*), sappan wood, the bark of Amur cork tree, etc. [4]

Apart from the choice of ingredients, the ratio of the mixture, in particular the ratio of glue to pigment, was also of particular importance on the final product and what was desired [6]. The recipes vary in their specifications and miscellaneous ingredients a sign that a lot of

experimentation took place. Furthermore, ink masters of old China were very careful to keep their recipes secrets [6].

Ancient Chinese inks remain a culturally charged but also mysterious product that have been particularly studied in recent years using new available analytical instruments.

Up to now, microscopy and surface chemistry still remain the main methods used to study traditional and ancient inks, in particular differences in particle size and aggregation among inks of different soot origin and presence of animal glue [3,8–12].

However, the study of ink from ancient manuscripts with the aim of characterization requires the use of other analytical techniques able to identify compounds characteristics of the different ingredients entering its composition. Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) has become a versatile technique commonly used in the domain of cultural heritage where it was employed for the characterization of different materials: wood, resins, gums, varnishes, papers, as well as modern and ancient inks. To the best of our knowledge, only a small number of Py-GC/MS study for the analysis of ink compounds are available in the literature [1,13–16], and no publication so far have investigated the composition of ink from ancient manuscripts.

For this study, we proposed to use for the first time a Py-GC×GC-MS approach, that can provide high chromatographic peak capacity, separation, and lower detection limit for the analysis of small molecules, thus allowing the identification of trace components in complex mixtures [17], to characterize inks from ancient manuscripts.

This study was performed in two steps. In a preliminary step, to test the performance of Py-GCxGC/MS in the characterization of inks, we analyzed reference samples of traditional Chinese inksticks. The analytical approach was then applied to four Tocharian manuscripts of the Pelliot Collection stored in the *Bibliothèque nationale de France (Paris, France)*. The manuscripts dated to the second half of the 1st millennium CE were discovered in ruins of Buddhist sites, in the Xinjiang region in the West of China. They are all written in the Tocharian B language and belong to a relatively circumscribed area, to wit the region of Kucha, in the Tarim basin, on the northern fringe

of the Taklamakan desert.

2. Materials and Methods

2. 1 Material

In the hope to identify characteristic compounds of inks, in addition to referring ourselves to ancient texts sources and literature, this work incorporated the analysis of several reference samples. For this study, we chose 1) a traditional black Chinese inkstick (from the old Hu Kai Wen ink workshop 金不换 Jin Bu Huan 净烟 Jing Yan black inkstick) made from industrially produced oil soot and labelled as blackHKW, 2) a traditional red Chinese inkstick from the old Hu Kai Wen ink workshop and labelled as redHKW, 3) a traditional Chinese black inkstick of unknown composition from the “Chinese Brush Painting Set" of Jane Dwight and labelled as blackJD, 4) a sample of pine soot ink , previously analyzed in our laboratory in the course of previous works, made from the soot of pine wood which is produced by the anoxic burning of pine wood and labelled as blackIP. While these traditional Chinese inksticks can be acquired easily, the secret of their manufacture is often well guarded, in particular regarding the animal glue and additives used in their fabrication.

To overcome this sample limitation, references samples stored in the Center for Research on Conservation (CRC) of beef collagen, goat collagen and ox gelatin often reported as being used in ink manufacture were also analyzed in two replicas. Tiny samples of pork gelatin, fish gelatin and deer glue, more difficult to get hold of, were also analyzed in this study. Finally, samples of traditional handmade Kozo paper (*Broussonetia kazinoki sp.*) were also analyzed as control samples and used as a paper support for the analysis of reference ink samples. Most of the analyses of reference samples were conducted in duplicates when possible.

Typical quantities of sample used in this work ranged from 70ug for reference samples up to 150ug in the case of the manuscript samples with ink.

Regarding the ancient manuscripts, four Tocharian B manuscripts from different periods

(according to the relative chronology of the script), and found in different sites near Kucha, were sampled and analyzed for this study [18]: 1) two literary texts - PK AS 12M (6^{th} century CE, Duldur-Akhur) and PK AS 7F (7^{th} century CE, Duldur-Akhur), 2) one magical text - PK AS 8B (7^{th} century CE, Subashi) and 3) one Vinaya text (set of Buddhist monastic rules) - PK NS 95 (7^{th} century CE, Duldur-Akhur). They are written in various styles of the Tocharian Brāhmī script. Two tiny fragments per manuscript were sampled: one fragment without visible ink present and one fragment of manuscript with ink, with the objective on identifying with certainty compounds issued from inks from compounds that could be introduced by the making process of the paper.

2. 2 Method

Pyrolysis was performed using a Frontier Lab pyrolyzer PY-3030iD directly connected to the injection port of the gas chromatograph. Samples were placed in a stainless-steel sample cup on top of the pyrolyzer at near ambient temperature and dropped in the pyrolysis tube into the pyrolysis chamber. The temperature of the interface was held at 320 °C. The sample cup was introduced into the furnace at 500°C, the chosen temperature for the analysis of the paper samples.

The GCxGC-MS analyses were conducted with a Shimadzu QP2010-Ultra mass spectrometer equipped with a two-stage thermal modulator ZX 2. The GC injector, was operated in split mode at a split ratio of 30:1 and set at 280°C. The GCxGC setting utilizes two columns: an OPTIMA-5HT column (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used as first dimension column and a Zebron ZB-50 (2.8 m × 0.1 mm I.D., 0.1 μm film thickness) was used as a second-dimension column. Effluent from the first column is trapped in the modulator for a given period (modulation time) before being focused and released into the second column. In the case of compounds that coelute from the first column, the second column may allow for separation due to the different polarity of the stationary phase.

The two columns are connected in a serial fashion using a micro-union column connector. The separation was carried out at an initial constant pressure of 300 kPa (which resulted in a flow rate

varying from 1.0 mL/min to 0.4 mL/min during the acquisition) and using Helium Alphagaz 2 as carrier gas. The ZX 2 two-stage thermal modulator employs a closed cycle refrigerator/heat exchanger to produce a ca.−90◦C cooled air jet regularly modulated with a pulsed hot air jet.

The optimized parameters were set up on the basis of the results of the analysis of reference samples. The optimized modulation period was 9s with a programmed hot pulse of 0.350 s. A two-step temperature program was used for the hot jet set at 200°C for 30 min and subsequently raised to 280°C in order to increase the modulation step efficiency in correlation with the volatility of pyrolysis products. The oven temperature was initially held 1 min at 100°C, and then ramped at 2°C min−1 to 325°C, where it was held for 25 min, for a total program time of 138.50 min. The mass spectrometer was set to scan 50 to 600 m/z, using electron ionization at 70 eV. The MS interface was kept at 300°C and the ion source at 200°C.

Data processing of the Py-GCxGC/MS raw data was achieved using GC Image software, version 2.4. The chromatogram obtained through repeated trapping and injection is rendered in two dimensions, with the first and second dimensions on respective axes. The identification of the various unknown compounds required the subtraction of the background signal from the mass spectra before identification performed by comparing the mass spectra of unknown components with the ones of the NIST mass spectra library, 2011 edition. Data treatment using GC Image provides four pieces of information for each compound: the first-dimension retention time (1tR), the second-dimension retention time (2tR), peak volume and peak value. Before having access to this information, peaks must be detected by the 'blob detection' function via GC Image software. The blob detection algorithm implements a post-processing filter to eliminate detected peaks/blobs that have either an area (number of included pixels) that is too small or a maximum peak value that is too small. After manual verification of the chromatographic peaks, blob detection was done by setting the following parameters in GC Image: minimum area (number of included pixels) for peak detection of 20 for data analysis, as well as a default minimum peak of 3.0 times the estimated standard deviation of the noise. It is important to consider that by setting the threshold to a low area

for peak detection, it might result in a large number of detected chromatographic peaks with a high rate of unknown identifications.

To look at compounds' distribution, we used the blob volumes, meaning the sum of the values of the pixels in the blob, representative of the relative abundances of each compound in the sample analyzed and determined by GC-Image. Percentages of compounds detected in each analysis were calculated considering the ratios of each compound of interest to the sum of all the considered compounds.

All statistical analyses and figure preparations were performed using RStudio, version 1.4.1106. Regarding clustering analysis, the default Euclidean distance measure was used. Furthermore, the two more common linkage functions, complete and single, were evaluated based on their cophenetic correlation coefficient, which is a measure for how well the dendrogram preserves the pairwise distances between the original data points [19]. The complete linkage method generated the strongest cophenetic correlation ($c > 0.85$), indicating that this method best preserves the pairwise correlations.

The results of hierarchical cluster analysis can be illustrated in a tree diagram, called a dendrogram. A dendrogram comprises of lines that connect clusters. The bottom row of a dendrogram consists of leaves, which represent the individual samples included in the cluster analysis. A cluster is usually defined as two of more leaves that are joined by a common node and a branch. The height in a dendrogram represents the distance between two samples or clusters [19].

3. Results and Discussion

3.1 Principal components of reference inks

The most significant components identified in our two samples of black inks of reference are polycyclic aromatic compounds (PACs), in particular polycyclic aromatic hydrocarbons (PAHs*)*, characteristic of soot. The PAHs identified range from $C_{14}H_{10}$ to $C_{19}H_{14}$. As previously mentioned,

PAHs isomers give very similar EI spectra so to distinguish them you will need to verify their retention times (1rT and 2rT).

PAHs content have been used to differentiate wood soot versus lampblack/carbon black. Lampblack, traditionally produced by collecting soot from oil lamps is a subtype of carbon black produced by the incomplete combustion of heavy petroleum products (such as tar) or vegetal oil. From Wei *et al*., statistically, amounts of PAHs emanating from pine soot was presented as higher than those emanating from lamp soot and proposed as a criterion for their differentiation [14]. In accordance to this observation, our sample of 52ug of blackIP made with pine soot present a higher PAH content in comparison of our samples of blackHKW (69ug and 70ug) made with manufactured oil soot (Figure 1). Moreover, in a previous study the relative content of benzo[k]fluoranthene was proposed as a criterion to differentiate the two types of soot, presenting benzo[k]fluoranthene as higher in pine soot than in lamp soot. However, our experiments demonstrate here that such differentiation deserves a more careful analysis of the content in PAHs.

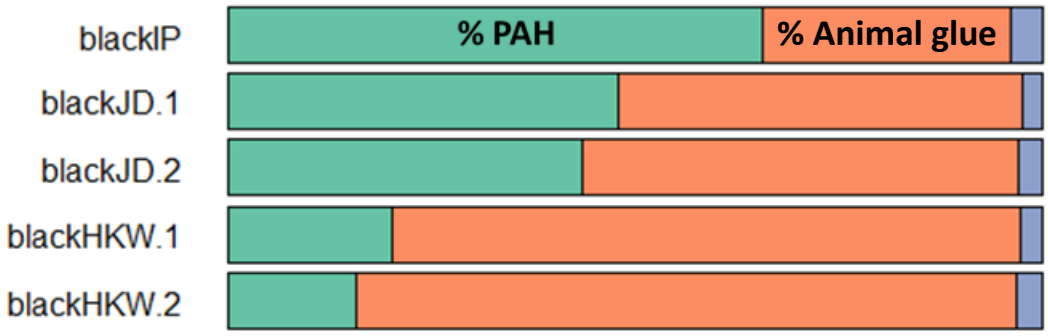

Figure 1: Percentage of *polycyclic aromatic hydrocarbons* (*PAHs*) (in green), compounds characteristic of animal glue (in red) and other compounds (in blue) in the sample of blackIP (52ug), blackJD.1 (67ug), blackJD.2 (54ug), blackHKW.1 (69ug) and blackHKW.2 (70ug).

Compared to our samples of blackHKW and blackJD, the sample of blackIP present a higher quantity of lower PAHs (2-4-ring PAH) than higher PAHs (5-6-ring PAH) characteristic of softwood soot (Figure 2). A large literature has investigated the formation, occurrence, and distribution of PAHs in order to source the types of wood and combustion conditions [20–23]. However, it is still

unclear, if wood type-specific PAH patterns in soot exist [24], particularly because combustion conditions can have a big impact on PAH formation. Orasche *et al*. [25] and others have noted that the higher the temperature in the absence of oxygen, the larger the PAH molecules that are formed, and at lower temperatures, the occurrence of smaller PAH increases [26]. Furthermore, comparison of the PAH distributions with literature data is difficult due to several reasons: 1) sampling and combustion conditions, including ovens types, which may widely differ in the studies may have, as previously said, a significant impact on the formation of PAHs; 2) focus of the studies vary and thus PAH analyzed and presented differ depending of the studies resulting in poor comparability particularly for cultural and ancient artefacts, and finally 3) employed wood types vary according to locations worldwide [24]. However, it has been presented that softwood (coming from gymnosperm trees, usually evergreen conifers, like pine or spruce) and hardwood (coming from angiosperm — or flowering plants) could be differentiated by looking at the amounts of higher PAHs (5–6-ring PAH) in contrast to lower PAHs (2-4-ring PAH) [24]. In the soot of the softwood, 2–4-ring PAH are in higher quantities whereas in soot of the hardwood, the content of 5–6-ring PAH was higher. However, as mentioned before, it is possible that the 2–4-ring PAH concentrations are low because they de-volatilize when the wood burns at a higher temperature or they were never formed in the first place [25]. Yet, there are some light polycyclic aromatic hydrocarbons that are resistant to high temperature. While fluorene, phenanthrene, benzo[a,h]anthracene, and chrysene emissions significantly decreased around 1000°C, indeno[1,2,3-cd]pyrene, naphthalene, acenaphthylene, fluoranthene, pyrene, and benzo[a]pyrene have been shown to be resistant to an increase in temperature [27]. It is also significant to look in details at methylated and cyclopenta-fused PAHs as their presence has been presented as possible markers of wood soot versus carbon black [28]. Finally, Oxygenated PAC such as 9,10-Anthracenedione have been also identified in wood combustion samples, such as in the combustion of pine wood [29]. However, methylated and cyclopenta-fused PAHs as well as Oxygenated PAC have been detected in all three black inksticks (Table 2 in Supplementary material). Finally, 3-ring PAH retene detected solely in our sample of

BlackIP is often reported as a product of wood combustion found in the emission particles particularly in softwoods like pinewood, oak and eucalyptus since softwoods are highly resinous [30,31].

Contrary to the traditional black Chinese inksticks, redHKW did not present any PAHs apart from retene and thus has not been considered further for soot characterization. While soot was and is still used today in ink fabrication in particular for black inkstick, other minerals could have been used in the fabrication of this red ink, such as cinnabar/vermillion (mercuric sulfide—HgS) or red ochre (iron oxides) [7].

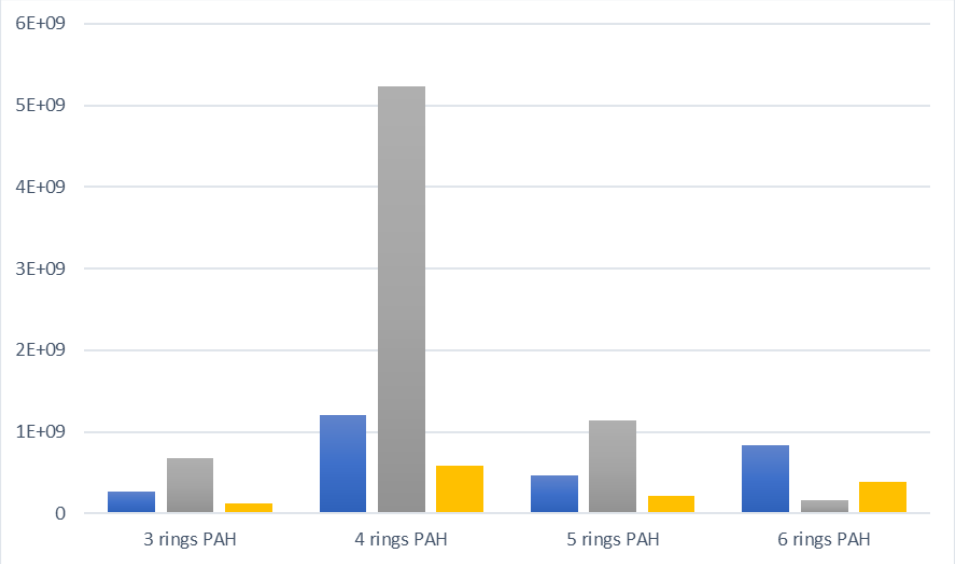

Figure 2: Relative quantity of 3 to 6 rings *PAHs* in the three reference samples of traditional Chinese inksticks: blackJD (in blue), blackIP (in grey), blackHKW (in yellow).

Compounds characteristic of animal glue were also detected in the inkstick samples analyzed.

Animal glue is a traditional material used extensively as adhesive and binder obtained by boiling skin, connective tissue and bones from mammals and fish. As fresh, these animal parts consist of collagen, a fibrous, insoluble protein, which degrades into gelatin when the protein is suspended in water and heated. As mentioned before, glues from different parts of diverse animals have been used as part of the ingredients of ancient Chinese inks. To identify the compounds characteristic of

animal glue, several reference samples of animal collagens, gelatins and glues were chosen based of textual information [4] and accessibility.

As already demonstrated in Cersoy et al. [32], the potentialities of Py-GCxGC/MS in term of complex mixture separation greatly enhanced the detection of markers of animal glues, in particular through the mapping of numerous diketopiperazines (DKPs) [32,33]. Fourteen DKPs have been identified in our reference samples of inks. Other small aromatic compounds were also identified such as pyrrole derivatives, which can be proposed as likely markers for animal glue [34] (Table 1 in Supplementary material).

The challenge of identifying specific markers in order to strengthen the identification of proteinaceous materials is an open issue in the scientific community particularly in face of time and degradation [34]. The first interrogation lies in the definition of compounds which can be assigned as reliable markers of such materials during the pyrolysis analysis. If DKPs are clearly attributable to such an origin, e.g. pyrrole ring derivatives are difficult to attribute unequivocally to such materials in view of the various materials able to be thermally degraded in these structures. To circumvent these drawbacks, the most efficient approach resides in comparative analysis of reference materials suspected to be present in the unknown samples; this is such a strategy that will be followed here to track the presence of animal glues of different origins in the reference inks and extended to the manuscript samples.

Initially, analysis and treatment of data relative to the detection of protein material markers were conducted in the aim of checking the possibility to differentiate the animal's origin of the reference glue, gelatin, or collagen samples by Py-GCxGC/MS. Looking at the experimental results (Figure 3), it seems that we are primarily differentiating the extraction procedures of collagen materials (ox, pork, goat, etc.) rather than differentiating the native collagens. For instance, differences between collagen and gelatin consist in a degree of hydrolysis of the native collagens in the associated gelatins, this hydrolysis being able to modify the association of amino-acid chains during the formation of the gelatins. Consequently, differences in expected sequences of amino acids present

in the native collagen chains of different animal species can be diminished, or even cancelled, by the hydrolysis process during the preparation of corresponding gelatins. Under such conditions, and considering such a possibility, it is not surprising that the observed clustering of ox gelatin and beef collagen are not associated and to observe the grouping of ox gelatin and pork gelatin. On a side note, the addition of pork gelatin in ox gelatin is a reasonable possibility to be considered for the commercial preparation of ox gelatin.

Apart from ox and pork gelatins presenting similar profiles with high relative concentration of Cyclo(Pro-Gly) and Cyclopentadecane and are difficult to differentiate on the basis of DKPs and associated proteinaceous marker present, the classification of the samples presented in Figure 3 conducts to the discrimination of deer glue and fish gelatin from ovine and bovine collagen and gelatin. While deer glue and fish gelatin are on the same branch, the high height of the link (around 8) which represents the distance between the two samples demonstrates that they have distinctive profile (see pie charts in Figure 3). Moreover, bovine collagen, presenting fewer proteinaceous compounds and higher relative concentration of pyrrole derivatives, is very dissimilar from the rest of the reference samples (Figure 3 and Table 1 in Supplementary material).

Each reference sample presents therefore a distinctive profile that could be interesting to compare to the ink sticks reference samples.

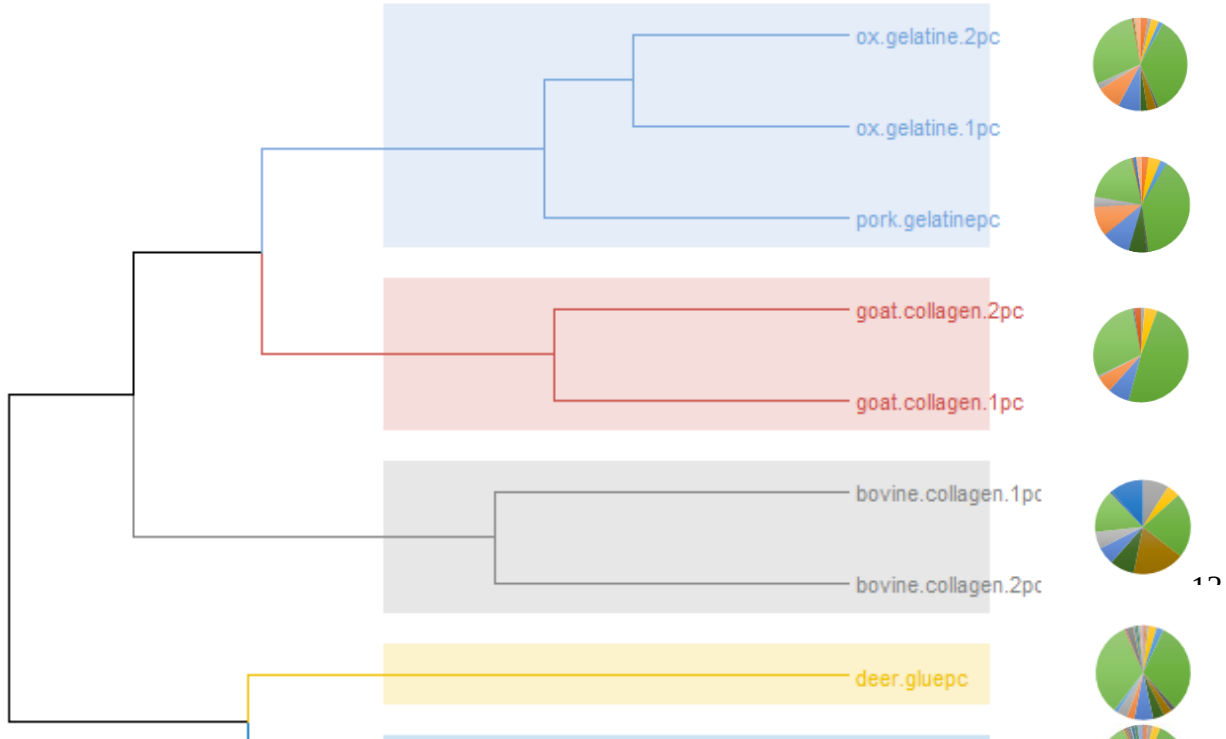

Figure 3 : Dendrogram of the hierarchical clustering of proteinaceous compounds in our samples of collagens, gelatines and animal glues using scaled data and complete as the linkage type (c = 0.86), accompanied with pie charts representing proteinaceous compounds distribution using peak volumes for each samples (see list of compounds in Table 1 of Supplementary material).

Aware of limitation of the comparative strategy followed for the classification of reference ink samples considering the animal origin of glues, the observed tendency demonstrate however that when comparing data with our samples of inksticks (Figure 4), the sample ink blackJD present the most similarities with fish gelatin, corroborated with the detection of a high percentage of Cyclo(Pro-Gly , Cyclo(L-leucyl-L-phenylalanyl) and Cyclo(Leu-Leu) (Table 1 in Supplementary material). It is more difficult to pronounce ourselves regarding the animal glue that would have been used for the sample ink blackHKW. As for the sample ink blackIP, the clustering analysis isolates it from our reference samples (i.e., farthest away from all). As demonstrated above through the characterization of PAHs and PACs, the inkstick blackIP was likely made from pine soot and present less glue (30%) than the sample ink blackJD (53%) and the sample ink blackHKW (81%) made from oil soot ink. It is thus entirely possible than another type of binder not encompassed in this study would have been used in its manufacture.

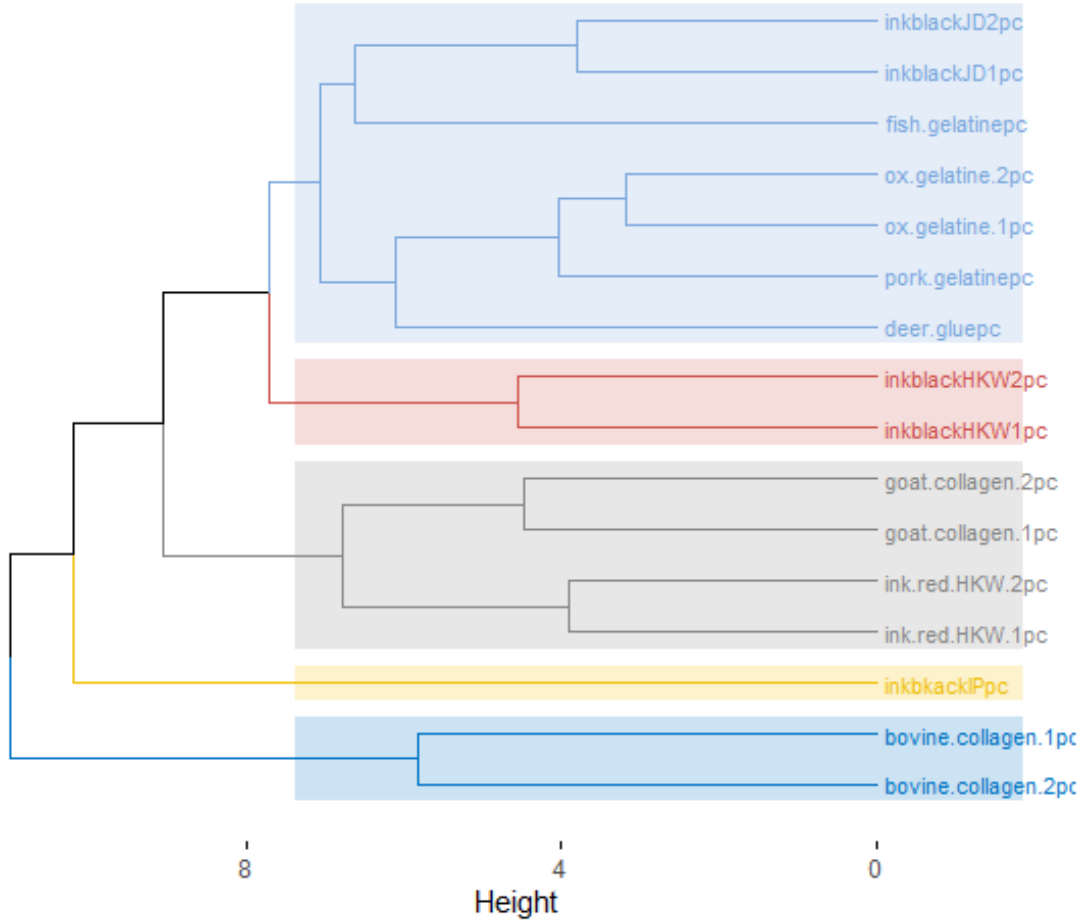

Figure 4: Dendrog
references using sc
1 in Supplementary

Other compounds have been identified in our three inksticks of reference demonstrating the potential of the approach based on Py-GCxGC/MS for the characterization of sample's components. A list of these components is reported in Table 3 – Supplementary materials. While we were unable to trace the possible origins of some of these compounds, several of them could be attributed to additives expected to be detected in ink sticks considering previous studies [13,14] and the historical data available [1-7]. Those additives can be very varied, hence the difficulty of chemically identifying them with certainty. These compounds could be coming from additives added to color - or improve the color of - the ink, to perfume it or for microbial protection purposes. For instance, borneol and camphor, identified in blackHKW, were likely used for their antimicrobial and aromatic properties as mentioned in previous studies [14]. As mentioned above, retene is characteristic of

conifer wood soot. However, previous studies state that the presence of retene when dehydroabietic acid derivatives characteristic of *pinaceae* resins [35] are also detected - like it is the case in redHKW - could be the evidence of conifer tar [13].

In redHKW, we were also able to identify the compound Murrayaquinone a (compound 3.5 in Table 3 in Supplementary material). It is a carbazole alkaloid that has been isolated in the essential oil from plants from the genus Murraya (*Rutaceae*) [36]. Their shrubs are very fragrant, and their fruits are orange, red, or black and could have been used for their anti-microbial properties or/and to either perfume or enhance the red color of the ink. It is interesting to note that several pyrolysis products previously reported to be common to different classes of characteristic synthetic pigments [37] as for example, aniline and carbazole, which are commonly used in the synthesis of pigments, were found exclusively in the red ink sample.

In addition to help us set the optimal protocol parameters for the analysis of manuscript samples, the use of reference samples allows us to assess the performance of the Py-GC×GC/MS in the detection and identification of characteristic compounds of inks, as well as test and evaluate the repeatability of our analysis (i.e: variability in the detection of characteristic compounds) that will be impossible to evaluate with our samples of ancient manuscripts from the Pelliot collection.

3.2 Py- GC×GC/MS data: compounds detection and data repeatability

This work also examines the performance of Py-GC×GC-MS for ink composition study by analyzing the compounds profiles of reference samples, in particular small quantities of traditional Chinese black inkstick as such or applied on the reference kozo paper using an ink stone to grind up the pigment and then a small quantity of distilled water to produce a liquid then applied on kozo paper using a bamboo reed pen.

The experimental results showed that the number of compounds of interest detected by Py-GCxGC-MS was ca. 170 peaks for the analysis of traditional inks and 220 peaks for the analysis of

traditional inks on paper (Figure 5). Furthermore, ca. 20% of the compounds were not successfully identified precisely. However often the class of chemical compound (e.g. alkanes, fatty acids, etc.) could be at least determined with certainty. Furthermore, those compounds revealed to be in fact mostly structural isomers can be for the most part distinguished using their retention times (1tR and 2tR): this case is well illustrated in the case of PAHs.

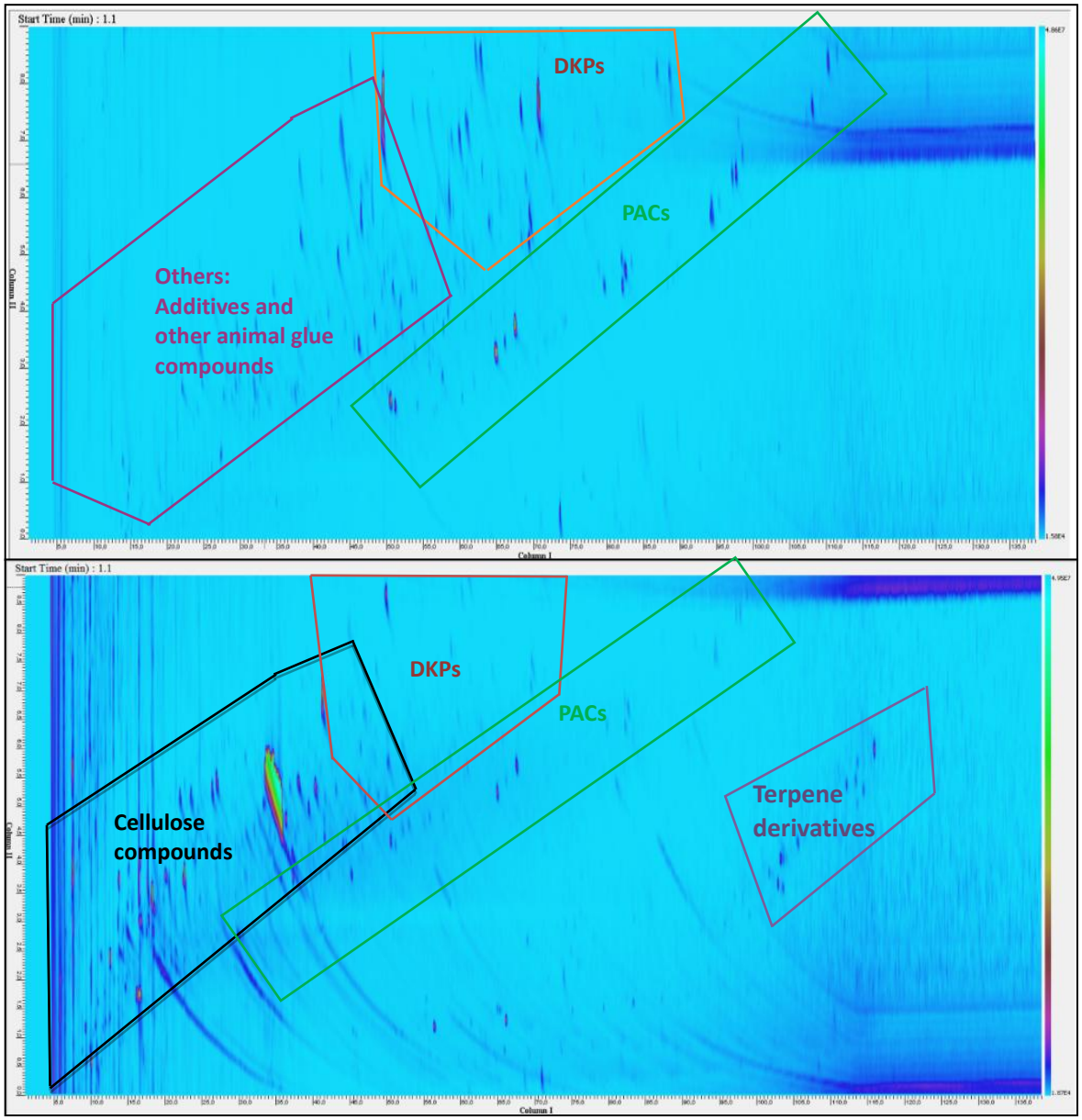

Figure 5: a) Py-GC×GC-MS Pyrogram from a sample of the traditional black inkstick "blackJD" (54ug of sample), b) from a sample of reference Kozo paper (90ug) with a quantity of ink estimated at ca. 3ug of the traditional Chinese inkstick of blackJD applied on it (93ug of sample).

Moreover, the Py-GC×GC-MS data set show little variation in the distribution of compounds from one analysis to the other. Sample of inkstick at our disposal were analyzed in duplicates to look at the repeatability of our analysis, which will have a great impact on our interpretation of the manuscript samples.

The two samples of inkstick show a good repeatability from one analysis to the other when looking

for example at the distributions of polycyclic aromatic hydrocarbons (PAHs) (Figure 6.1, a and b) and compounds attributed to the presence of animal glue (Figure 6.2, a and b).

This study was also interested in examining possible loss of information through the dilution of characteristic ink components when ink is applied on a paper. The results are presented in Figure 6, c and d. The presence/absence and global distribution of PAHs clearly varies very little when samples of inksticks (Figure 6.1, a and b) and samples of the same ink applied on a reference Kozo paper were analyzed (Figure 6.1, c and d). We can particularly note in Figure 6.1 the regularity in the distribution of the main PAHs: phenanthrene, anthracene, 4H-Cyclopenta[def]phenanthrene, Fluoranthene, Benzo[ghi]perylene and Indeno[1,2,3-cd]pyrene (2.3, 2.4, 2.5, 2.6, 2.7, 2.30 and 2.31 in Table 2 of Supplementary material).

On the contrary, we observed some modification in the observed distribution of certain compounds characteristic of animal glue (Figure 6, c and d) and in particular the loss of nine compounds: 4 pyrrole derivatives, [1,3]Diazepan-2,4-dione, p-Cresol, picolinamide, 3-benzyl-6-isobutyl-2,5-dioxo-piperazine and L-Proline, 1-acetyl-. However, the main compounds of animal glue found in blackJD are still detected when applied on paper (1.6, 1.9, 1.10, 1.12, 1.13, 1.14, 1.15 and 1.18 in Table 1 of Supplementary material). The fact that PAHs in lower relative concentration compared to these proteinaceous compounds are detected indicates that the disappearance of these nine proteinaceous compounds is not due to their concentration but rather seems to be inherent to their nature. Moreover, the same nine compounds detected in our sample redHKW were also lost in our sample of redHKW applied on paper (Table 1 in Supplementary material). While this study cannot give the reader answers on the reasons explaining the disappearance of these nine compounds, our data reveals the importance of taking into account this possible loss of information when interpreting the results of the analysis of inked manuscripts.

Furthermore, while it is not the case in our reference samples of Kozo paper, manufactured from the fibers of *Broussonetia kazinoki* with no sizing, sizing agents based on proteinaceous materials are expected as a ground layer applied to the paper before painting and inking in ancient East Asian

papers [39–41]. Pyrolysis products issued from sizing compounds are part of compounds regarded as likely detected during our analysis of the ancient manuscripts from the Pelliot Collection. Noticeably, proteinaceous material of different origins can, therefore, be present in the samples of inked manuscripts as adhesive in a ground layer of the manuscript (sizing) and as binding media during inkstick manufacture. As it is difficult to distinguish firmly the origin of the markers emanating from those proteinaceous materials of different use, the assignment of origin of the relevant compounds could constitute an issue in data interpretation of the analysis of ancient inked manuscripts. One way of circumventing this issue, and chosen in the following, lies in the comparative investigation of samples of the historical manuscripts probing part of them with ink and part of them without visible ink, to conclude on the most likely origin of characteristic pyrolysis products by comparison.

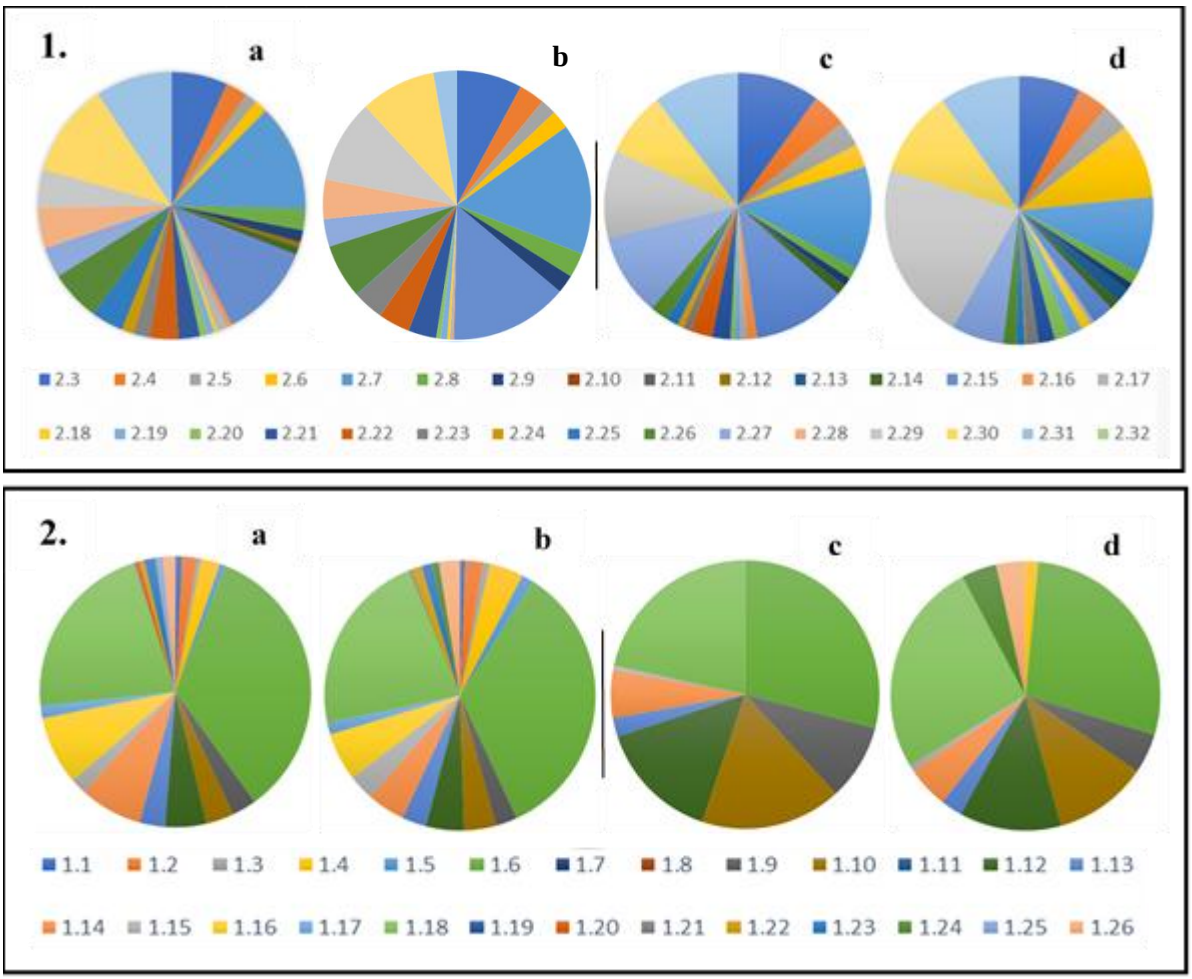

Figure 6: Pie charts representing the distribution using the blob volumes of 1) polycyclic aromatic hydrocarbons (PAHs) (PAHs ID 2.3 à 2.32 in Table 2 of Supplementary material) and 2) compounds characteristic of proteinaceous binder (ID 1.1 à 1.6 in Table 1 of Supplementary material) detected and identified by Py-GC×GC-MS in two samples of the traditional Chinese inkstick of blackJD (**a and b**), as well as two samples of Kozo paper with ca. 3ug of the same aforementioned ink applied on it (**c** and **d**).

3.3 Application to four Tocharian manuscript samples

Following the same experimental procedure than those applied for the reference samples of Chinese inks and animal glues, four manuscripts of the Pelliot Collection stored in the *Bibliothèque nationale de France* were selected for their representativeness and historical value.

Based on the results of the analysis of reference samples of inks, we can remark that two inks may have been manufactured with a similar pigment and similar additives but with different

proteinaceous binders. In order to study similarities/difference in ancient inks' production, it is thus important to look further than global composition and examine in detail the components characteristic of each main constituents of inks: soot, animal glue and additives. These manuscripts were therefore analyzed for the detection of characteristic PAHs, proteinaceous marker compounds, and likely additives able to enlighten similitude in composition of their inks and in techniques employed in ancient ink making during a period spanning from the 6^{th} to 8^{th} century CE in the Tarim basin (Xinjiang, China).

With the objective of discriminating with certainty compounds issued from inks from compounds that could be introduced by the making process of the paper, as specified above, comparison of pyrograms of inked manuscripts and manuscript fragments without any visible ink present were made using the software ImageJ and JPEG image of the pyrograms. The images were first converted into 8-bit gray scale images and then two different colors were attributed to each image using the RGB colorscale. Finally, the two images were stacked together to reveal difference in presence/absence of compounds of interest. Figure 7 reports the result of comparison between the samples associated to manuscript PK AS 8B (Figures 1-3 Supplementary material report the results for manuscripts PK AS 7F, PK AS 12M, and PK NS 95 respectively).

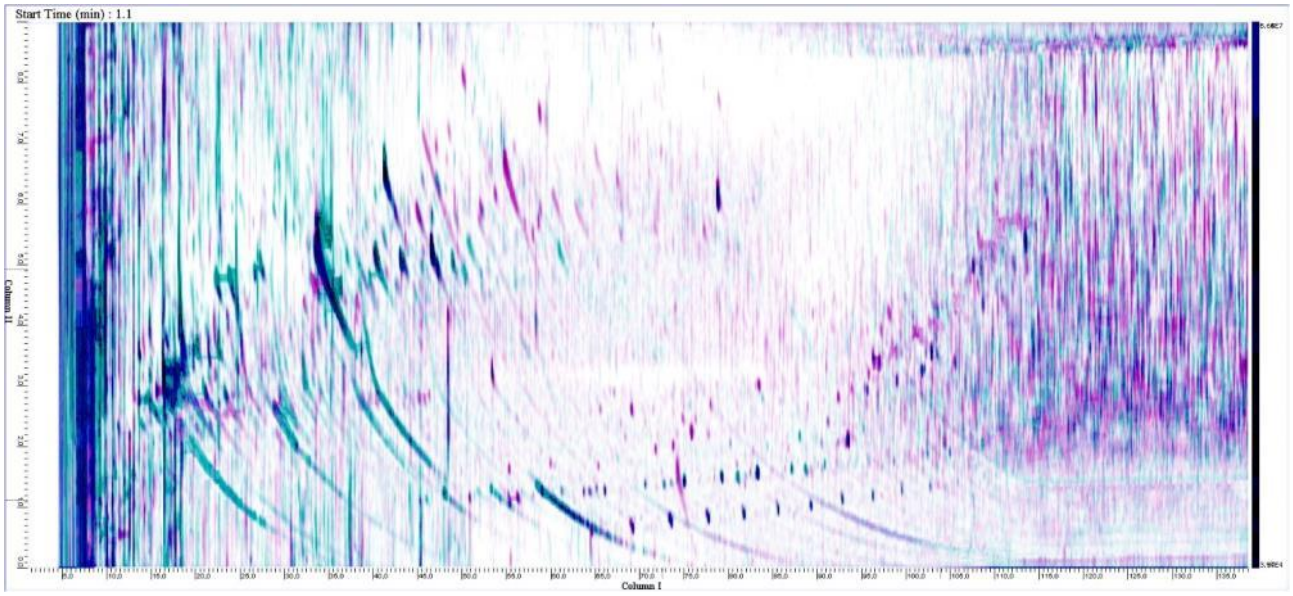

Figure 7: Comparison using the software ImageJ of the pyrograms for the fragment without ink (blue) and with ink

(pink) of samples PK AS 8B, highlighting the compounds of interest (see text).

As shown in these stacked images, several PAHs have been identified in the four manuscripts fragments (Table 2 in Supplementary material). They all point to the use of softwood soot for the manufacture of the inks of the four manuscripts

First, the detection of several methylated and phenylated PAHs (9H-fluorene, 9-methyl-, anthracene 2 methyl, naphthalene, 1-phenyl-, naphthalene, 2-phenyl-, pyrene, 1-methyl- and pyrene, 2-methyl-) is in accordance with a wood soot origin for these two manuscripts and not with a carbon black or lampblack origin [28].

The main PAHs detected in all four manuscripts are 3-5 rings PAHs: such as fluorene, anthracene, fluoranthene, pyrene, pyrene, 4,5-dihydro-, benz[a]anthracene, benzo[c]phenanthrene, benz[e]acephenanthrylene, perylene, benzo[b]fluoranthene, benzo[a]fluoranthene, and retene, pointing to the use of softwood soot in the inks for all four manuscripts [24]. The relative percentages of those 3-rings, 4-rings and 5-rings PAHs are reported in Figure 8 for the two religious texts PK AS 12M and PK AS 7F, the Vinaya text PK NS 95 and the magical text PK AS 8B, respectively.

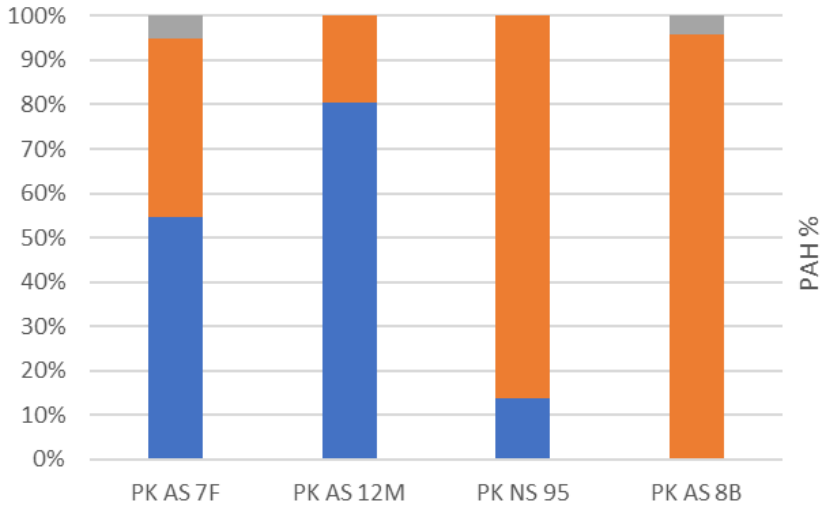

Figure 8: Percentage of 3-rings (blue), 4-rings (orange) and 5-rings (grey) PAHs in the four samples of manuscripts PK AS 7F, PK AS 12M, PK NS 95 and PK AS 8B.

PK AS 7F and PK AS 12M, in particular, present similarities in their PAHs distribution that points to the use of pine soot. First, the high quantity of 3-rings PAHs, such as fluorene and anthracene, but most importantly retene [29,42]. The presence of retene detected in our fragments of PK AS 7F, PK AS 12M is particularly significant as this alkylated three-ringed PAH is a known conifer burn product [21,22,30,31], which can therefore be considered as a reliable clue for the likely assignment of origin of soot employed in these ancient inks.

To confirm the conifer PAH signature in the manuscripts PK AS 7F and PK AS 12M, we used the Ret/3-ring ratio [Ret/(Ret + Phen + Ant)] presented in Karp et al. [20]. Indeed, as reported in this study, Ret/3-ring ratio highlights the source signal of conifers while accounting for the potential influence of burn phase, transport, and biodegradation. The study shows that angiosperm samples had consistently low Ret/3-ring values (<0.1), while gymnosperm values were higher, but ranged widely (0.2–0.8). PK AS 7F and PK AS 12M present Ret/3-ring values of 0,37 and 0,28 respectively and thus confirm the pine soot signature.

On the other hand, the fact that retene was not identified in the manuscript PK AS 8B while its PAH signature points to the presence of softwood soot could be explained by the use of a different wood. However, different methods of making pine-soot cannot be ruled out, in particular combustion conditions have been mentioned as having a significant impact on the formation of PAHs [25,26].

To reinforce this identification, comparison with reference samples of traditional Chinese inkstick shows that the four manuscripts are forming a different branch than our reference samples of ink blackJD and ink blackHKW made from oil soot when treated statistically following a hierarchical clustering analysis (Figure 9). Moreover, as a confirmation of the results of comparison of the PAHs ratios, clustering analysis confirmed that PK AS 12M and PK AS 7F present the most similarities with ink blackIP made from pine soot (Figure 9).

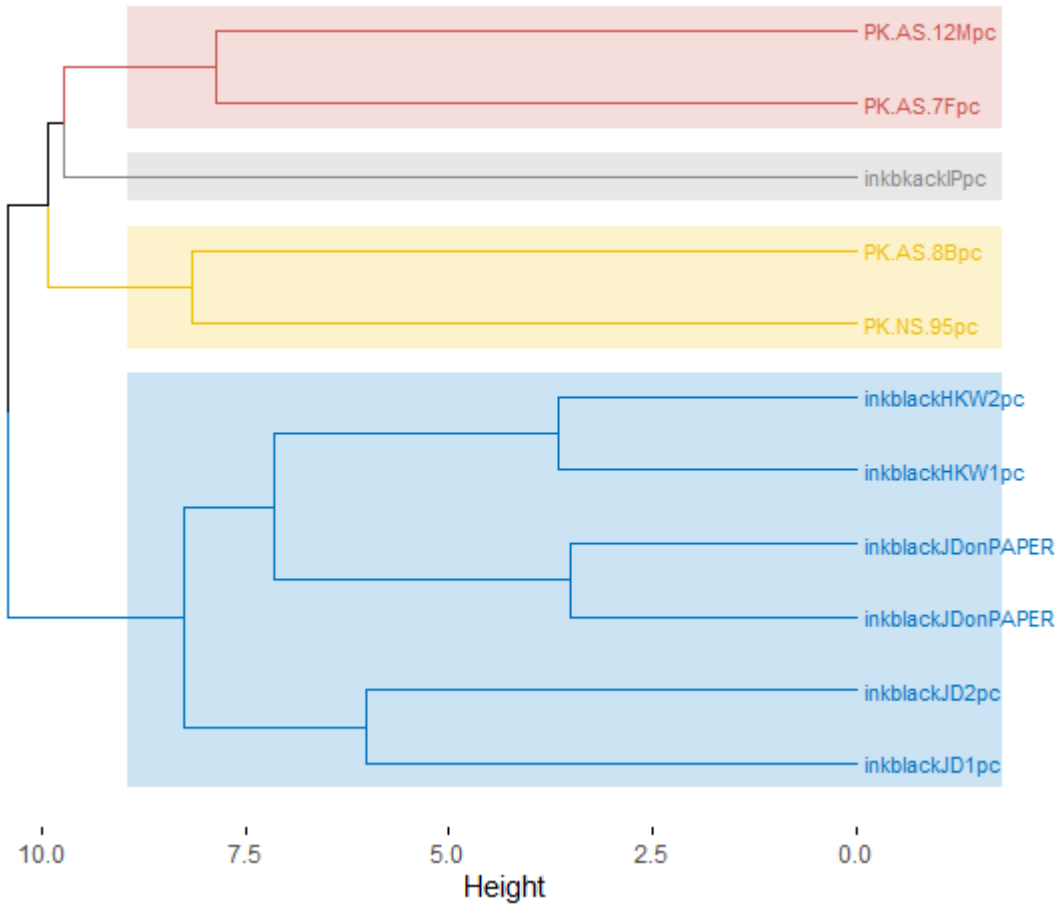

Figure 9: Dendrograms of the hierarchical clustering of PCA in our reference samples and manuscripts samples using scaled data and complete as the linkage type (c = 0.91) (see list of compounds in Table 2 in Supplementary material).

Regarding the marker compounds characteristic of proteinaceous binders investigated above, apart from PK NS 95 for which nine DKPs have been identified, the other three manuscripts presented seven of the fourteen DKPs previously mentioned.

Looking at the distribution of DKPs and other compounds attributed to the presence of proteinaceous binders, inks in PK AS 12M, PK AS 8B and possibly PK AS 7F seem to have been made with a similar type of animal glue as a result of their clustering together (Figure 10). In comparison, the ink from PK NS 95 could have been made with a different type of animal glue due to its separation from the three previous ink samples in the dendrogram (Figure 10). Moreover, when we look at the percentages of each constituent (PACs, proteinaceous binders, and additives) for each inked manuscript, PK NS 95, in particular, presents a high relative concentration of

proteinaceous material (Figure 11) compared to PK AS 7F and PK AS 12M and thus could be another indication of a different ink manufacture.

However, as put into light in part 3.2, misleading interpretation of the Py-GCxGC/MS analysis can result from the fact that certain compounds attributed to animal glue can be found as adhesive in ground layers of manuscripts as well as binding media in inkstick manufacture. For instance, three of the DKPs (Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a:1',2'-d]pyrazine) can be identified in our fragments of PK AS 7F and PK NS 95 sampled without visible ink by binocular magnifier (Table 1 and Figures 1 and 3 in Supplementary material). Thus, we cannot discard the possibility that some of the DKPs detected here could be coming from both the paper sizing and the ink composition.

Moreover, the difficulty to characterize specifically the animal glue likely used in the fabrication of the ancient inks can be rationalized in three ways: 1) another type of animal glue was used in the manufacture of the inks than those utilized as reference samples, 2) the samples of manuscripts could have degraded and could now present pyrolytic profiles irrespective of the nature of the proteins present [34] or 3) we are in the presence of a mix of proteinaceous material attributed not only to the ink but also to the paper.

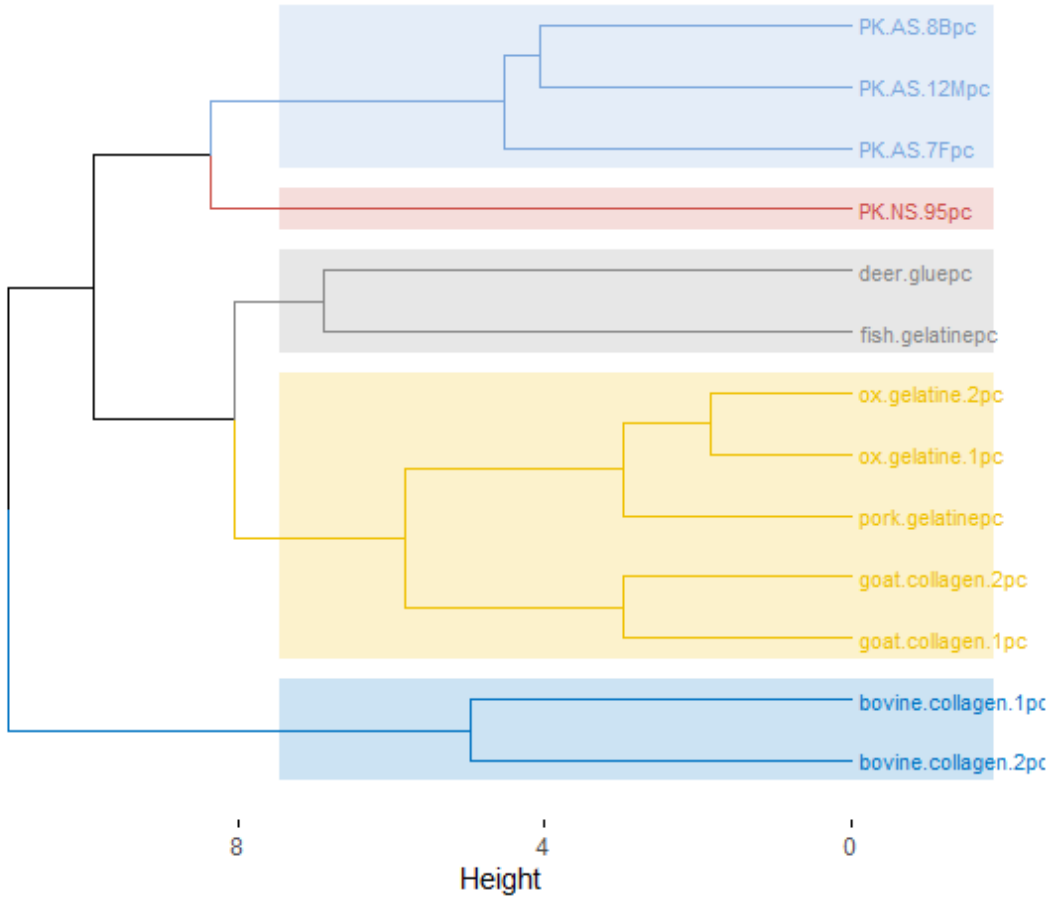

Figure 10: Dendrograms of the hierarchical clustering of animal glue compounds in our reference samples and manuscripts samples using scaled data and complete as the linkage type (c = 0.86) (see list of compounds in Table 1 in Supplementary material).

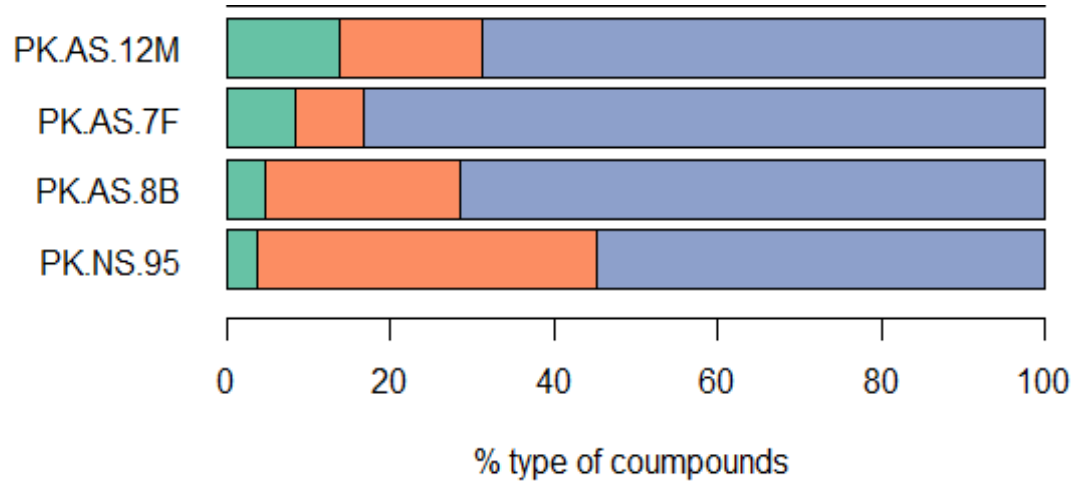

Figure 11: Percentages of animal glue compounds (green), PACs (orange) and compounds attributed to additives (blue) in each fragment of manuscripts.

Finally, several other compounds attributed to additives have been detected and identified solely in our fragments with inks. Possible origins of these compounds are proposed considering previous studies [13,14] and the historical data available [1-7].

No compounds indicating the presence of the additives mentioned in previous studies on Chinese inksticks from earlier periods (such as borneol, camphor or cedar oil) [1,13] have been detected in our four manuscripts. However, as said before, retene has been identified in PK AS 7F, PK AS 12M and PK NS 95 with 7-Oxodehydroabietic acid, methyl ester, a typical oxidation product of a resin from the Pinaceae family [31,35,43] and thus could be attributed to the addition of conifer tar [13]. However, methyl dehydroabietate and dehydroabietic acid usually expected, were not detected to corroborate this assumption [1,13,15]. Indole, also mentioned in these studies, has been identified in PK AS 7F and PK NS 95. This compound has been attributed to the presence of eggs white [13,15]. However, no other compounds such as 3-methyl-1H-pyrrole and 3-methyl-indole respectively were detected, to back up this theory [15] and since it has been identified in the fragments without visible ink, we cannot discard the possibility that indole is coming from the manuscripts.

The detection of phenolic derivatives as 2-methoxy-4-vinylphenol and phenol-2,6-dimethoxy in the pyrograms of PK AS 7F, PK NS 95 and PK AS 8B (Table 3 in Supplementary material) have been attributed in a previous study on Armenian medieval to 19th century ink recipes [16] to the presence of peels of pomegranate which is rich in polyphenolic compounds [44]. Pomegranate peel has been used as one of the ingredients of Chinese inks during the Tang dynasty (618 – 907 CE) [4]. Furthermore, the accumulation of dibenzofuran, 4-methyl-, pyroglutamic acid and β-D-Glucopyranose, 1,6-anhydro- detected in fruits and pomegranate peels strengthen this assumption [44]. However, phenol-2,6-dimethoxy and β-D-Glucopyranose, 1,6-anhydro- (levoglucosan) are currently identified as lignin and cellulose markers of paper [39,45,46] and could be in fact coming from the manuscript.

PK AS 8B presents three compounds not detected in the three other manuscripts: β-sitosterol acetate, Ursa-9(11),12-dien-3-yl acetate, Lupeol acetate. These compounds are identified separately

in several plants but together solely from few sources, like in the wood bark of *Moraceae* (Mulberry tree) [47]. Moreover if Mulberry tree has been largely used to produce Chinese papers [4], microscopic analysis of the manuscripts conducted in the course of the HisTochText project stated a hemp or ramie origin of the paper used for writing the Tocharian B manuscripts of the sample [48], consequently, one can suspect that the three previous compounds could be attributed to ink composition rather than its paper material. These three triterpenoids have been also identified in the genius *Salvia*, the sage herbs [49–51]. While mentions of medicinal herbs and incense used as additives in ancient Chinese inks such as clove, comfrey, ash (*Fraxinus chinensis*) bark, sappanwood and sandalwood –, can be found in historical data [2,4], no evidence of the addition of sage herbs has been found so far to corroborate this theory.

4. Conclusions

This work demonstrates the potential of Py-GCxGC/MS in the analysis and study of ancient ink on tiny fragments of ancient manuscripts of historical value. Thanks to this approach, we were able to put into light similarities and differences in inks manufacture otherwise invisible or hardly discernible in details through other analytical techniques, making this approach highly relevant to Cultural Heritage.

In an initial phase, the analysis of reference samples of traditional Chinese inks allowed us to identified characteristic chemical signatures of soot and binding agents as well as possible additives. In addition to demonstrate the validity of the method, the analysis of reference samples of traditional Chinese inks allowed us to evaluate the repeatability of this approach (i.e: variability in the detection of characteristic compounds) from one analysis to the others, that would have been impossible to test otherwise on historical samples,

The results demonstrates that the presence/absence and global distribution of PAHs clearly vary very little when samples of inksticks and samples of the same ink applied on paper are analyzed. On

the contrary, we observed some modification in the observed distribution of certain compounds characteristic of animal glue. Our data reveals the importance to take into account this possible loss of information when interpreting the results of the analysis of inked manuscripts and a detailed explanation on the reasons behind this phenomenon, while outside the scope this study, deserves further analysis.

The results of the present study demonstrate that the Py-GCxGC/MS approach, and the gain in sensitivity and resolution inherent to the technique, enable the identification of characteristic PAHs signatures of soot and compounds characteristic of binding agents even on tiny amount of inked manuscripts samples. The results show that while the inks of the four manuscripts were produced using a similar soot made from the combustion of pine wood, diverse animal glues seem to have been used for their fabrication. For instance, the results for PK NS 95 indicate a different ink manufacture. This approach is also effective in the identification of various possible additives based on the cooccurrence of compounds pointing, for example, to the presence of conifer tar and pomegranate peel mentioned in the literature of ancient Chinese ink. More evidence is now needed, using historical data available, for a secure identification of these additives. Finally, it is important to note that the Py-GCxGC/MS approach give us access to an important information: the relative mixing ratio of soot and glue that could give us further indication on the fabrication of the ink and its quality [52].

CRediT authorship contribution statement:

E. P.: Formal analysis, Validation, Investigation, Visualization, Writing - original draft, Writing - review & editing. **G.-J. P.**: Funding acquisition, Project administration, Resources, Writing - review & editing. **M. S.**: Conceptualization, Supervision, Writing - review & editing,

Declaration of Competing Interest:

The authors report no declarations of interest.

Data Availability Statement: All data derived during the experiments are given in the paper.

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