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# Paper fragments from the Tibetan Samye Monastery: Clues for an unusual sizing recipe implying wheat starch and milk in early Tibetan papermaking

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## ABSTRACT

The origin of Tibetan papermaking is poorly documented. Up to now, only the Tibetan paper fragments from Endere and Dunhuang sites have been scientifically studied outlining the basic knowledge about plant fiber uses and artwork manufacturing procedures. In order to deepen the knowledge on the unique ancient Tibetan papermaking, we report here the analysis of a set of paper fragments from the Samye Monastery in south Tibet, China. Multidisciplinary scientific investigations utilizing <sup>14</sup>C radiocarbon dating, microscopic observation, scanning electron microscopy (SEM), pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), pyrolysis-comprehensive gas chromatography/mass spectrometry (Py-GCxGC/MS), and proteomic analysis have been applied to these ancient paper fragments. The results show that the fragments were dated to the 12<sup>th</sup>~13<sup>th</sup> century AD and made from fibers of the *Thymelaeaceae* family plant *Stellera Chamaejasme* L of which the fiber's chemical profiles have been firstly studied. The presence of starch granules from *Triticum spp* wheat and proteins from bovine milk (more likely assigned to native yak), offers the first indication that a unique sizing technique using starch-milk sizing had been employed in early Tibetan papermaking, compared to using alum-animal glue sizing agent which is more popular in contemporary Central China. The combined use of organic material (unaltered starch grains) and inorganic material (calcium carbonate) as paper fillers is also firstly revealed in Tibetan papermaking. The application of Py-GC/MS, Py-GCxGC/MS and proteomic analysis revealed more detailed profiles of early Tibetan hand papermaking, indicating that these methods have great potential to explore the production technology of ancient handmade papers.

Keyword: Tibetan paper, *Stellera Chamaejasme* fiber, sizing technique, starch-milk sizing, casein, Py-GC/MS, proteomics

## 1. Introduction

Historical records of Tibetan hand papermaking are rare, discontinuous, and subject to divergent discussions [1, 2]. The early known records could be traced back to the Tang Dynasty in the Book of Tang·Tubo biography (Tubo represents the Tibetan regime in ancient China), which reports that the representatives of Tubo (649AD) asked Tang Dynasty dignitaries for the help of the craftsmen of paper and ink making, which was approved by Emperor Gaozong (628-683AD) of Tang Dynasty. Correspondingly, the date, 650AD, has been marked by scholars as the beginning of Tibetan paper making [3]. Afterwards, unfortunately, reports were absent for thousands of years until Qing Dynasty (*e.g.*, 1732AD) when written documents reporting data on Tibetan hand papermaking reappeared [4].

Tibetan hand papermaking craft is one of the main features of Tibetan culture due to its exotic features of manufacturing and use, different from those of central China [5-7]. The inaccessibility, as well as the mystery surrounding Tibetan hand papermaking, has attracted the attention of Europeans explorers during early journeys to Tibet Plateau [8]. In compensation for rare written descriptions, the remains of Tibetan paper fragments provide us a way to unravel the mysterious of its craft upon scientific analysis. As currently known, the earliest Tibetan remains were unearthed in Endere Temple, located out of Tibet Plateau in now Xinjiang Autonomous Region (Fig.1) by Marc Aurel Stein in the 1900s [9]. Stein discovered 27 pieces of tough and yellowish paper leaves with Tibetan writings, known now as the oldest specimens of Tibetan writing of eighth century [10-12]. The microscopic examination of these fragments by Professor J. Wiesner has attributed its fiber origin to a *Thymelaeacea* plant, namely *Daphne cannabina* Loar with high probability [13]. As this plant and their kindred species are not known to grow locally in Endere (Fig.1), it was then reasonable to deduct that the fragment of concern was not locally made but imported from Tibet [12]. Attributing the origin of plants in Tibetan paper samples is a key point in the knowledge of the techniques first used since the beginning of traditional plant papermaking in ancient Tibet.

Other sources for the study of ancient Tibetan fragments out of Tibet Plateau come from the Dunhuang manuscripts that were found at the beginning of the 20<sup>th</sup> century from the famous ‘library cave’ at Dunhuang (Fig.1, modern Gansu Province, China). These manuscripts stored now in different museums have been, for some of them, the subject of investigations under the angle of textual, palaeographical or codicological studies combined to paper analysis using microscopy and spectroscopy [1, 14]. On the basis of these experimental results, the paper type was aligned to its geographical origin with the threefold classification of origin around Central Asia, central Tibet or eastern Tibet [1]. These results confirmed the variety of papermaking methods and potential plant materials used in correlation with the availability of local plants for papermaking, which does not simplify the definition of a ‘Tibetan paper’. Moreover, it has appeared that depending on the function of the objects: texts, paintings, ritual items, banners, technological choices in paper and pigments could be made [15]. However, these regional features may reflect existing communication and exchanges overtaking limited areas,

characterization of which needs further investigations for completion. Currently, it is admitted that palaeographical analysis is more useful for dating the manuscripts, and paper's analysis is more relevant to determine their geographical origin [1].

In the process to study ancient Tibetan fragments, the analytical methods for the materiality study was proved to be an effective method to reveal the material and crafts involved in its making. Methods such as microscopic fiber analysis [1], FTIR [16], XRF [14, 15, 17], immunological techniques [18] have been applied for Tibetan fragments scientific examinations. Recently, the capacity of a chromatographic based method for the material study of East Asian handmade papers has been explored [19, 20] as well as a method of higher selectivity (comprehensive gas chromatography GCxGC) with a more quick and reliable identification procedure [21-23]. The criterion of the set chromatographic base method can get a detailed chemical information of the paper components, focusing on plant fibers, as complementary to the information gained so far by microscopic analysis [20].

Currently, there are few reports on the study of ancient fragments collected in the Tibetan regions or presented in different museums [16-18, 24] while the dedication of scholars for the study of ancient Tibetan manuscripts has greatly improved our knowledge for history and development of hand papermaking in ancient Tibet [25]. Nevertheless, there are still undiscovered points about its rich connotation and culture that deserve to be investigated. Consequently, in view of advances obtained in the analysis of East Asian papers through chromatographic applications, new projects on ancient paper fragments surveys and scientific analyses were carried out in order to deepen our understanding of the ancient papermaking craft.

Samye Monastery is located in the center of Samye in Dranang County, Shannan prefecture of Tibet, China (Fig.1). It is regarded as the first Buddhist monastery on the Tibet Plateau, which was under construction during 12 years under the patronage from the Tubo Trisong Detsen in the eighth century [26]. It is currently one of the most valuable monasteries in Tibet and is listed as Key Cultural Relics Protected Sites (KCRPSs) in China. Fragments of ancient papers pertaining to this study was discovered in Samye Monastery which was part of Prajñāpāramitā Hrdaya (the Perfection of Wisdom). These fragments provided an opportunity to reveal the early hand papermaking in ancient Tibet which suffers from little documentation.



Figure.1 the location of Endere, Dunhuang where ancient Tibetan papers were discovered and the location of Samye Monastery in south Tibet, China.

The research reported here provided the opportunity to explore the materiality and making crafts of ancient Tibetan handmade paper fragments preserved within the oldest monastery on Tibet Plateau. These fragments were scientifically examined following a multidisciplinary approach based on microscopic analysis, radiocarbon dating, pyrolysis-gas chromatography/mass spectrometry and proteomic analysis under a microinvasive protocol for sampling. The experimental results were discussed under the angle of their interest and archaeological meaning leading to the improvement of our knowledge on paper composition and recipes of early Tibetan papermaking.

## 2. Materials and methods

Here, we addressed a number of key points related to ancient paper fragments analysis by carrying out the following analyses:

- Sample collection to study ancient Tibetan paper and compare different papers made with *Thymelaeaceae* family fibers.
- Radiocarbon dating to ascertain the date of Samye manuscript samples.
- Microscopical analysis in order to characterize the fiber origin.
- Py-GC/MS and Py-GCxGC/MS analysis to confirm the origin of fiber plant material as well as the presence of additives.
- Proteomic analysis to determine the origin of animal materials and starch origin in the sample.

### 2.1 Sample collection

Several paper fragments composed as part of Prajñāpāramitā Hrdaya (the Perfection of Wisdom) were discovered within Samye Monastery during a cultural heritage survey and two fragments were submitted for scientific analysis (Fig.2). Materials used as modern references of *Thymelaeaceae* sp. consisted of handmade paper from *Stellera chamaejasme* prepared following the traditional Tibetan papermaking processes as well as two other types of papers made from *mitsumata* (*Edgeworthia papyrifera* Sieb. & Zucc.) and *gampi* (*Wikstroemia sikokiana* Franch. & Sav.) made with Japanese traditional processes.



Figure.2 the paper fragments preserved in the Samye Monastery.

## 2.2 $^{14}\text{C}$ radiocarbon dating

A piece (about 10 mg in weight) of paper was cut from the clean part of the ancient Tibetan fragment. The surface sheets were first washed in distilled water with an ultrasonic cleaner and then treated with 1.2N HCl and 1.2N NaOH solutions (60-70 °C). After re-treating with 1.2N HCl and rinsing with distilled water, they were combusted using CuO (850 °C, 3h) to form CO<sub>2</sub>. The CO<sub>2</sub> was reduced to graphite by H<sub>2</sub> (650 °C, cat-Fe, 6h). The radiocarbon ages were measured by AMS facility in Nagoya University. Another piece was cut and send to be dated by accelerator mass spectrometry (AMS) at the Beta Analytic Radiocarbon Dating Laboratory (Miami, Florida, USA). The dating procedure followed the strict protocols of the chosen laboratory. According to the  $^{13}\text{C}/^{12}\text{C}$  ratio obtained, the measured date was corrected to the conventional age ( $^{14}\text{C}$  yr before present; BP) [27, 28].

## 2.3 Microscopic observation

Direct microscopic observation of the fragment surface was conducted with a digital microscope (VHX-600, Keyence). The observation was conducted in reflection mode. For fiber analysis, a small amount of fibers from the clean part of the fragment was taken with cleaned dissecting needles, and placed on the surface of a microslide with a drop of distilled water to disperse the fibers. The reagent of iodine-zinc chloride stains was then added and then covered with a cover glass. The prepared slide was then observed through Stereo Microscope. Scanning electron microscopy was conducted on a Phenom XL coupled with EDS system. For this analysis, the paper specimens were put on carbon adhesive tape for SEM observation. The SEM observation was conducted with 15 Kv, 20% spotsize and EDS analysis was conducted with 15 Kv, 75% spotsize in a low vacuum mode.

## 2.4 Py-GC/MS and Py-GCxGC/MS Analysis

Py-GC/MS analysis was performed using a vertical micro-furnace-type pyrolyzer PY-2020iD (Frontier Lab, Fukushima, Japan) directly connected to the injection port of a Shimadzu QP2010 gas chromatograph linked with a Shimadzu QP2010Plus quadrupole mass spectrometer

(Shimadzu, Champs-sur-Marne, France). The sample was placed in a stainless-steel sample cup. The paper samples were weighted with an XP2U Ultra Micro Balance (Mettler Toledo, Viroflay, France). Typical quantities of reference paper used in this work for the development of the analytical method ranged from 80 to 110  $\mu\text{g}$  weighted with the microbalance. For the balance's capacity used (2.1g), readability and repeatability of the microbalance were 0.0001 and 0.0002 mg, respectively. The sample cup was placed on top of the pyrolyzer at near ambient temperature. The sample cup was introduced into the furnace at 500  $^{\circ}\text{C}$ , and then the temperature program of the gas chromatograph oven was started. The Py-GC interface was held at 320  $^{\circ}\text{C}$ . Chromatographic separation was carried out on an Agilent DB5-ms 5% phenyl-95% dimethyl polysiloxane fused silica capillary column (30 m length, 0.25 mm inner diameter and coated with a 0.25  $\mu\text{m}$  film thickness). The oven temperature was initially held 3 min at 40  $^{\circ}\text{C}$ , and then ramped at 5  $^{\circ}\text{C min}^{-1}$  to 325  $^{\circ}\text{C}$ , where it was held for 10 min. The total duration of GC analysis was 70 min. The helium carrier gas Alphagaz 1 (Air Liquide, France) was used in the linear velocity mode (1 mL  $\text{min}^{-1}$ ). The injector was held at 280 $^{\circ}\text{C}$  and used in split mode (1:10 of the total flow). The mass spectrometer was operated at 5000 u.s $^{-1}$ , with a scan range from 42 to 500 u, using electron ionization at 70 eV. The interface was kept at 300  $^{\circ}\text{C}$  and the MS source at 200  $^{\circ}\text{C}$ . The mass spectra of the pyrolysis products were recorded using the average spectrum + average and subtract spectrum modes in the Shimadzu GCMSsolution software. Identifications were achieved on the basis of EI mass spectra by interpretation of the main fragmentations [22] and using the NIST MS library (2011).

Py-GCxGC/MS analysis was conducted with a Shimadzu QP 2010 Ultra mass spectrometer (Shimadzu, Champs-sur-Marne, France) equipped with a two-stage thermal modulator ZX 2 (Zoex, Houston, USA). Pyrolysis was performed using a vertical micro-furnace-type pyrolyzer PY-3030D (Frontier Lab, Fukushima, Japan) directly connected to the injection port of the gas chromatograph. The sample cups and the paper samples were weighted similarly than during Py-GC/MS experiments. A sample of 70  $\mu\text{g}$  was weighted here. The sample cup was placed on top of the pyrolyzer at near ambient temperature. The sample cup was introduced into the furnace at 500  $^{\circ}\text{C}$  and the temperature program of the gas chromatograph oven was started. The pyrolyzer interface was held at 320  $^{\circ}\text{C}$ . An Optima-5HT column (30 m  $\times$  0.25 mm I.D., 0.25  $\mu\text{m}$  film thickness, Macherey-Nagel, Hoerd, France) was used as first dimension column and a Zebron ZB-50 (2.8 m  $\times$  0.1 mm I.D., 0.1  $\mu\text{m}$  film thickness, Phenomenex, Le Pecq, France) was used as a second-dimension column and for the loop modulator system. The separation was carried out at an initial constant pressure of 300 kPa in a constant pressure mode (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 (Air Liquide, Bagneux, France). The ZX 2 two-stage thermal modulator employs a closed cycle refrigerator/heat exchanger to produce a -90  $^{\circ}\text{C}$  cooled air jet regularly modulated with a pulsed hot air jet. The optimized modulation period for two-dimensional chromatogram collection was 9 s with a programmed hot pulse of 0.350 s. A multi-step temperature program was used for the hot jet set at 200  $^{\circ}\text{C}$  for 30 min and subsequently raised to 280 $^{\circ}\text{C}$  until the end of the acquisition. The oven temperature was initially held 1 min at 100  $^{\circ}\text{C}$ , and then ramped at 2  $^{\circ}\text{C}$



min-1 to 325 °C, where it was held for 25 min. The total duration of GC analysis was 138.5 min. The injector was held at 280°C and used in split mode (1:30 of the total flow). The mass spectrometer was operated at 20,000 u.s<sup>-1</sup>, with a scan range from 50 to 500 u, using electron ionization at 70 eV. The interface was kept at 300 °C and the ion source at 200 °C. Data processing of the 2D raw data was achieved using GC Image software, version 2.4 (Lincoln, Nebraska). Identification of components was performed by comparing the mass spectra of unknown components with reference compounds from the NIST MS library (2011) and interpretation of the main fragmentations.

## **2.5 Proteomics Analysis**

The fragmented sample was extracted with a filter-aided sample preparation protocol (FASP) modified from references [29-31]. Then extracted peptides were analyzed using tandem mass spectrometry (MS/MS). Peptides were loaded on a Thermo-Dionex Ultimate 3000 HPLC system interfaced with a Thermo Orbitrap Fusion Lumos mass spectrometer. The analytical column was Acclaim PepMapTM RSLC C18 capillary column (75 µm ID, 150 mm length; Thermo Fisher Scientific). Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid in 100% acetonitrile for channel B. The peptides were separated with a 135 min gradient elution at a flow rate 0.300 µL/min. Full-scan mass spectra were acquired in the Orbitrap with a m/z range of 300-1500 and a resolution of 120,000. Data-dependent MS/MS scans were acquired in an Orbitrap with a resolution of 15000 and 30% normalized collision energy in high energy collision induced dissociation (HCD) mode.

MS/MS datafile from the LC-MS/MS run were searched against the NCBI database using PEAKS X. The search parameters were as follows: the digest mode was set as semitrypsin; two missed cleavages were allowed; carbamidomethylation was set as fixed modification; deamidation (NQ) and oxidation (M) were set as variable modifications; precursor ion mass tolerance was 20 ppm and fragment ion mass tolerance was 0.02 Da. False discovery rate (FDR) was set at 5% and protein scores were filtered with  $-10\lg P \geq 20$  and  $ALC (\%) \geq 50$  (de novo only). In order to exclude the contamination from the lab, negative extraction control was performed and analyzed in parallel with the sample.

## **3. Results**

### **3.1 <sup>14</sup>C radiocarbon dating**

Paleography and codicology are effective methods in the examination and dating of old Tibetan paper or book fragments [1, 32]. The paleographical dating of the manuscripts corresponds to the date when the manuscript was drafted. It is usually assumed that the paper was consumed for manuscript drafting approximately upon its manufacture while the time span between the manufacture and utilization could also be large in considering paper storage and reuse. The

fragment found at Samye Monastery constitute part of Prajñāpāramitā Hṛdaya and was assumed to be dated from around the 13<sup>th</sup> century according to the paleography analysis. For confirmation of the origin, radiocarbon dating was conducted. The <sup>14</sup>C radiocarbon dating result indicates a calendar calibrated results of (1152-1260) cal AD (90.4% probability) and 1150-1219 cal AD (73.7% probability) by two independent radiocarbon dating laboratories (Fig.S1, Supplementary Information). The dating result showed agreement with the paleography analysis that the sample consists of a 12~13<sup>th</sup> century fragment. Although radiocarbon dating has proved to be an effective method to determine the written age of ancient Japanese documents [33], in the analysis of ancient Tibetan papers, most of the dating to date was conducted by paleography and codicology study while the first application of the radiocarbon dating method introduced in this study showed its efficiency and consistency in dating of ancient Tibetan papers in supplementary to paleography and codicology analysis.

### 3.2 Microscopic observation

Part of the fragment has undergone corrosion and discoloration at the edge while the middle part is in a comparatively better conservation state. Brown transverse scribal guidelines were clearly visible, which is an important feature of Tibetan hand written scrolls to distinguish handwritten specimens from the printed one (Fig.3a) [25]. The ink on the paper were submitted to different degrees of caking off as can be seen under microscopy observation (Fig.3). One can notice that because of the sizing (discussed in part 4.2), the ink dipped and agglomerated on the paper surface instead of diffusing and penetrating into the paper (*e.g.*, different from the unsized *Xuan* papers) (Fig.3). Raman spectroscopy analysis confirmed that the ink consists of  $\alpha$ -carbon black ink (Fig.S2, Supplementary Information) [34, 35].

In scientific analysis of paper artifacts, the starting point lies in the material origin itself which point to the plant fiber utilization. Paper fiber analysis is one of the most basic analysis method to obtain the paper components. It has been early observed that the local grown *Thymelaeaceae* family plants (*e.g.*, *Daphne* sp., *Edgeworthia* sp., *Stellera chamaejasme*) have been used for the manufacture of Tibetan paper from the evidence of field survey and ancient fragment analysis [8, 12, 25]. To distinguish *Thymelaeaceae* family plant through fiber analysis is a big challenge in which the fiber analysis for clear-cut identification has been observed only in limited cases. However, it has to be remind that only a few species within *Thymelaeaceae* family has been historically utilized for papermaking [36]. Among them, the *Stellera chamaejasme* specie which grows locally in Tibet Plateau can be a characteristic component for exclusive identification of Tibetan manufacturing papers [25]. Furthermore, the fibers of *Stellera chamaejasme* were observed to be more distinguishable from the *Daphne* sp. and *Edgeworthia* sp. within the *Thymelaeaceae* family: the fiber presenting ribbon-like shape with the impression of transparency [25]. This kind of transparent ribbon-like shape fiber was observed within the analyzed fragment (Fig.S3, Supplementary Information) that gives hints for its fiber origin as *Stellera chamaejasme*'s fiber. Further analysis based on Py-GC/MS analysis confirmed this assertion as reported below (Part. 3.3).



Figure.3 the state of the fragment under the microscopy: (a) the transverse scribal guidelines, (b) the ink diffusion state, (c) and (d) the agglomeration and caking off the ink on the paper surface.

### 3.3 Py-GC/MS analysis

In order to support the nuances observed for the morphological features of the ancient paper sample by microscopic observation, Py-GC/MS analysis of the Tibetan paper fragment and a modern reference sample of Tibetan handmade paper from *Stellera chamaejasme* was carried out. Noticeably, it was shown previously that paper material origin of traditional East Asian handmade paper can be deduced from Py-GC/MS analysis of the paper origin since fiber plants employed in the process of hand papermaking provide specific plant marker fingerprints correlated with the composition of the plant material in use [19]. Paper support represents one of the most important features of ancient unknown manuscripts, and potentially it can characterize their geographical region of origin. As reported above, microscopic investigations of Tibetan manuscripts found in several places in East and Central Asia have assigned different plant species in this aim by the analysis of paper fibers [1, 13, 14]. Among them, ramie (*Boehmeria nivea*), hemp (*Cannabis sp.*), paper mulberry (*Broussonetia sp.*), *Daphne* or *Edgeworthia sp.* were representatively observed [1]. As previously observed [20], hemp, ramie, paper mulberry-based papers have shown specific fingerprints in the region of interest (ROI) of their plant markers,

which permits to not consider them in our comparison here. At the opposite, *Thymelaeaceae* plants, plant family to which pertains the *Stellera sp.*, present an interest for the investigation of potential plant markers for assignment of fiber plant origin in the ancient Tibetan paper sample. For these reasons, a comparison between Tibetan paper sample from the Samye Monastery with a modern reference Tibetan paper sample was conducted as well as an extended comparison with a mitsumata (*Edgeworthia Chrysantha Lindl.*) paper sample and a gampi (*Wikstroemia sikokiana Franck & Sav.*) paper sample (both pertaining to *Thymelaeaceae* plant family).

The experimental results for the ROI of plant markers detected by Py-GC/MS are presented in Fig.4. As reported before [19], the chromatograms resulting from the pyrolysis of East Asian paper samples can be divided roughly into three main parts: the cellulose fingerprint, an intermediate region with hydrocarbons and fatty acid derivatives, and the marker part region which permitted the characterization of fiber origin designed as region of interest (ROI) for retention time above 45 min at the end of the chromatogram. Assignments of the plant markers observed in the ROI for the investigated Tibetan paper samples are reported in Table 1.

Table 1. Assignment of plant markers detected by Py-GC/MS in the ROI of the investigated Tibetan papers, modern reference paper sample and ancient paper sample from the Samye Monastery, with retention time ( $t_r$ ), main fragment ions, expected molecular weights, assigned formula and most likely attribution of the products.

No	$t_r$ (min)	fragment ions (in decreasing order of intensity)	MW	formula	assignment
1	49.11	95,123,93,81,107	412	NA	potentially epoxystigmastane structure
2	51.27	69,81,95,93,121	410	C <sub>30</sub> H <sub>50</sub>	squalene
3	53.21	81,145,105,95,121	396	C <sub>29</sub> H <sub>48</sub> /C <sub>28</sub> H <sub>44</sub> O	stigmasta-3,5-diene
4	54.31	105,91,79,93,95	398	C <sub>28</sub> H <sub>46</sub> O	NA (potentially stigmastane structure)
5	54.54	105,91,95,81,106	396	C <sub>29</sub> H <sub>48</sub>	isomer of stigmasta-3,5-diene
6	54.71	135,81, 95,143,119	394	C <sub>29</sub> H <sub>48</sub> O	stigmasta-4,6-dien-3-ol
7	54.91	141,142,81,128,95	378	C <sub>26</sub> H <sub>50</sub> O	NA (potentially stigmastane structure)
8	54.99	81,105,147,91,145	416	C <sub>29</sub> H <sub>52</sub> O	stigmastan-3-ol
9	57.06	136,121,95,79,93	412	C <sub>29</sub> H <sub>48</sub> O	stigmasta-5,7-diene-3-ol*
10	57.79	105,95,107,81,91	414	C <sub>29</sub> H <sub>50</sub> O	stigmast-5-en-3-ol, (beta-sitosterol)
11	58.96	174,91,159,410,81	410	C <sub>29</sub> H <sub>46</sub> O	stigmasta-3,5-dien-7-one
12	59.43	124,95,109,93,229	412	C <sub>29</sub> H <sub>48</sub> O	stigmast-4-en-3-one
13	59.82	43,136,95,55,81	410	C <sub>29</sub> H <sub>46</sub> O	stigmasta-4,6-diene-3-one

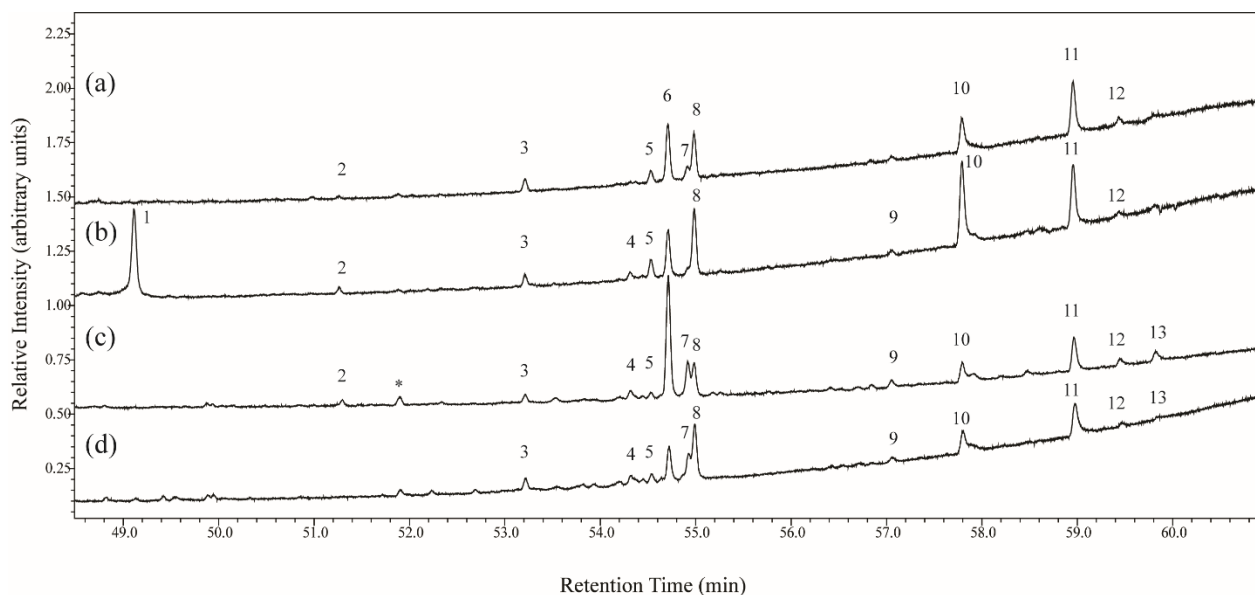
\* as previously reported [19] the fragment m/z 136 cast some doubt on this structure.

On one side, similarities appeared between the two Tibetan paper sample since the main differences between these two samples were observed through the detection of an additional peak

(#1, Table 1 and Fig.4) in the ancient Tibetan paper sample, and variations in the observed intensities of common markers with noticeable differences for some of them (#4, 7, 9, Table 1 and Fig.4). On the other side, a good correlation appeared between the two Tibetan paper sample (Fig.4a, 4b) and the two other samples of *Edgeworthia sp.* mitsumata (Fig.4c) and *Wikstroemia sp.* gampi (Fig.4d). Similitudes in the fingerprints of respective ROI were illustrated by the presence of stigmastane structures.

To go further in the evaluation of similitude of the ancient Tibetan paper sample and paper samples manufactured from *Thymelaeaceae sp.*, Py-GCxGC/MS analysis of a sample of the ancient paper and two sample of mitsumata and gampi papers have been carried out (Fig.S4, Supplementary Information). The resulting ROI for the three samples confirmed the similarities in distribution of marker compounds observed in Py-GC/MS with the advantage of a higher signal/noise ratio inherent to comprehensive gas chromatography as already observed with traditional East Asian paper [21].

With no doubt, the ancient Tibetan paper belonging to the Samye Monastery can be assigned to *Thymelaeaceae* plant species from the results of Py-GC/MS and Py-GCxGC/MS analysis. Moreover, the ancient Tibetan paper showed the characteristic fingerprint of a paper manufactured from *Stellera chamaejasme* as a plant material by comparison to the modern reference sample of handmade paper from *Stellera chamaejasme*.



**Figure 4.** Total ion current chromatograms of the regions of interest ROI obtained during Py-GC/MS analysis of (a) a reference modern Tibetan paper sample, (b) an ancient Tibetan paper sample from Samye Monastery, (c) a reference mitsumata paper sample, (d) a reference gampi paper sample.

### 3.4 Py-GCxGC/MS analysis

In the course of the Py-GCxGC/MS investigation of the ancient Tibetan paper sample from Samye Monastery, a close look has been taken on the peaks that could be attributed to protein

materials. Indeed, it appeared clearly that, compare to Py-GC/MS analysis, a two-dimensional analysis with its improved separation and its improved resolution increase noticeably the information content for the characterization of some targeted components in the ancient Tibetan paper sample. In particular, as previously noticed [37], a comprehensive gas-chromatography separation of the pyrolysis products allows a level of identification difficult to attain under monodimensional separation conditions for thermal degradation products of a protein content. The higher separation efficiency and the higher sensitivity represented the two main advantages of Py-GC×GC/MS analysis over Py-GC/MS analysis. A noticeable gain was so forth obtained for the diketopiperazine region (Fig.5).

The resulting Py-GC×GC/MS profile of the ancient Tibetan paper sample (Fig.5) is mostly characterized by (i) the cellulose fingerprint of the paper, (ii) the presence of diketopiperazines (DKP) issued from the thermal degradation of the amino acid chains of the protein content in the sample, (iii) a series of long chain alkanes/alkenes, the presence of fatty acid derivatives and other compounds (esters, acids), and (iv) a well-resolved region of interest ROI for the plant markers of *Stellera chamaejasme* (see above). Focus on these DKPs as well as protein markers could be made thanks to the GC×GC separation. The assignments of the main DKPs identified in the chromatogram as well as compounds identified as markers of casein are reported in Table 2. Among the detected DKPs, one can notice the presence of several DKP components previously detected in collagen sample [37], but nonetheless characteristic components of the collagen fingerprint are missing: Pro-Hyp, Gly-Hyp, Leu-Leu, Leu-Ileu. Moreover, intensities of Pro-Hyp DKPs are noticeably lower compare to a classical collagen fingerprint and additional presence of DKPs at MW 210, 228 were observed here. From these results, if the presence of collagen could not be totally eliminated, one can propose another protein content to rationalize the detection of the DKPs reported in Table 2.

Noticeably, markers of casein which have been previously characterized in proteinaceous binder in art material were detected during our Py-GC×GC/MS analysis [38, 39]. It was also observed that animal glue based on collagen addition presented a higher ratio of hydroxyproline during Py-GC/MS analysis since this latter amino-acid is more represented in collagen than in other materials like eggs or milk [38, 39]. Conversely, milk does not express much Hyp based DKPs as noticed here in the Py-GC×GC/MS experiments. To corroborate the Py-GC×GC/MS results, extracted ion chromatograms for the  $m/z$  154, 210 permitted to check the detection of traces of DKPs during the Py-GC/MS experiments, confirming so forth the assumption of protein addition in the recipe of hand papermaking potentially sourcing from milk.



381 Table 2. Assignment of DKPs and casein markers detected by Py-GCxGC/MS in the investigated Tibetan papers with retention time in  
 382 the first dimension (retention I) and second dimension (retention II), main fragment ions, expected molecular weights, assigned  
 383 formula and most likely attribution of the products. Presence of casein markers detected in [38] are reported in the last column.

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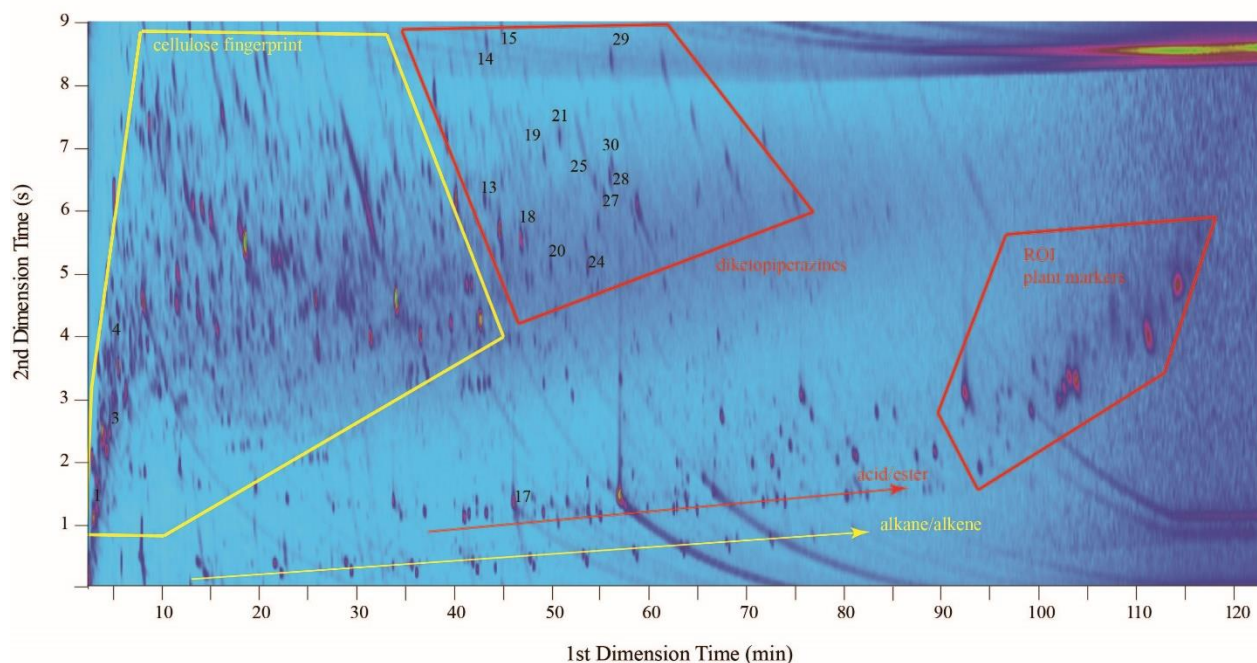
Compounds	Retention I (min)	Retention II (sec)	main fragment ions (in decreasing order of intensity)	MW	proposed formula	proposed assignment	Casein markers
1	2.9	1.40	110,53,109,5152	110	C <sub>6</sub> H <sub>6</sub> O <sub>2</sub>	2-Furancarboxaldehyde, 5-methyl-	x
2	3.95	2.15	126,55,69,83,111	126	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	Isomeric structure of isomaltol	
3	4.25	2.65	55,111,126,83,98	126	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	Ethanone, 1-(3-hydroxy-2-furanyl)- (isomaltol)	x
4	5.15	4.35	126,71,55,97,69	126	C <sub>6</sub> H <sub>6</sub> O <sub>3</sub>	4H-Pyran-4-one, 3-hydroxy-2-methyl- (maltol)	x
5	9.50	3.05	108,126,55,154,80	154	C <sub>10</sub> H <sub>8</sub> O	NA	
6	14.60	4.45	154,153,111,85,139	NA	NA	NA	
7	18.20	5.50	154,139,96,93,111	NA	NA	NA	
8	18.95	4.20	154,139,111,65,53	NA	NA	NA	
9	36.65	3.50	167,196,168,123,197	196	C <sub>10</sub> H <sub>12</sub> O <sub>4</sub>	NA	
10	36.80	3.35	54,70,137,151,108	NA	NA	NA	
11	39.80	6.15	182,181,111,183,96	182	C <sub>9</sub> H <sub>10</sub> O <sub>4</sub>	Benzaldehyde, 4-hydroxy-3,5-dimethoxy-	
12	42.65	6.15	186,93,65,130,64	226	C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> O <sub>4</sub>	Hyp-Hyp	
13	42.65	6.10	186,93,65,64,130	186		Glu-Gly, Asp-Ala, Ser-Val	x
14	42.95	8.60	70,69,97,68,168	168	C <sub>8</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Ala	x
15	44.45	8.85	70,69,97,168,68	168	C <sub>8</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Ala	x
16	45.05	4.90	181,210,91,182,121	NA	NA	NA	
17	45.95	1.15	111,83,70,69,154	154	C <sub>7</sub> H <sub>10</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Gly (+ co-eluting tetradecanoic acid MW 228)	
18	46.40	5.55	167,210,168,123,53	210	C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	NA	
19	48.80	6.85	70,154,72,125,69	196	C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Val	x
20	49.55	5.05	181,210,182,65,153	210	NA	NA	
21	50.45	7.15	70,154,125,69,72	196	C <sub>10</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Val	x

22	52.25	8.25	70,55,180,151,110	NA	NA	NA	
23	52.40	8.10	70,180,55,135,151	NA	NA	NA	
24	53.30	5.05	111,83,136,55,121	228	NA	Hyp-Asp, Ala-Val, Leu-Asp	
25	54.05	6.45	70,154,69,86,71	244	NA	Glu-Asp	
26	54.35	5.80	210,167,77,121,149	NA	NA	NA	
27	54.95	6.20	154,70,86,69,55	210	C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	x
28	55.55	6.50	70,154,125,69,55	210	C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Leu	x
29	55.70	8.35	70,194,69,96,68	194	C <sub>10</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	Pro-Pro	x
30	55.85	6.65	154,70,125,69,155	182	C <sub>11</sub> H <sub>18</sub> N <sub>2</sub> O <sub>2</sub>	Cyclo(leucylopropyl)	
31	58.40	6.05	210,167,77,154,149	210	NA	NA	
32	58.55	5.90	210,167,77,154,149	210	NA	NA	
33	68.75	6.35	186,117,116,90,228	NA	NA	NA	
34	73.4	6.15	186,117,91,187,57	NA	NA	NA	
35	74.6	6.15	186,117,118,90,187	NA	NA	NA	

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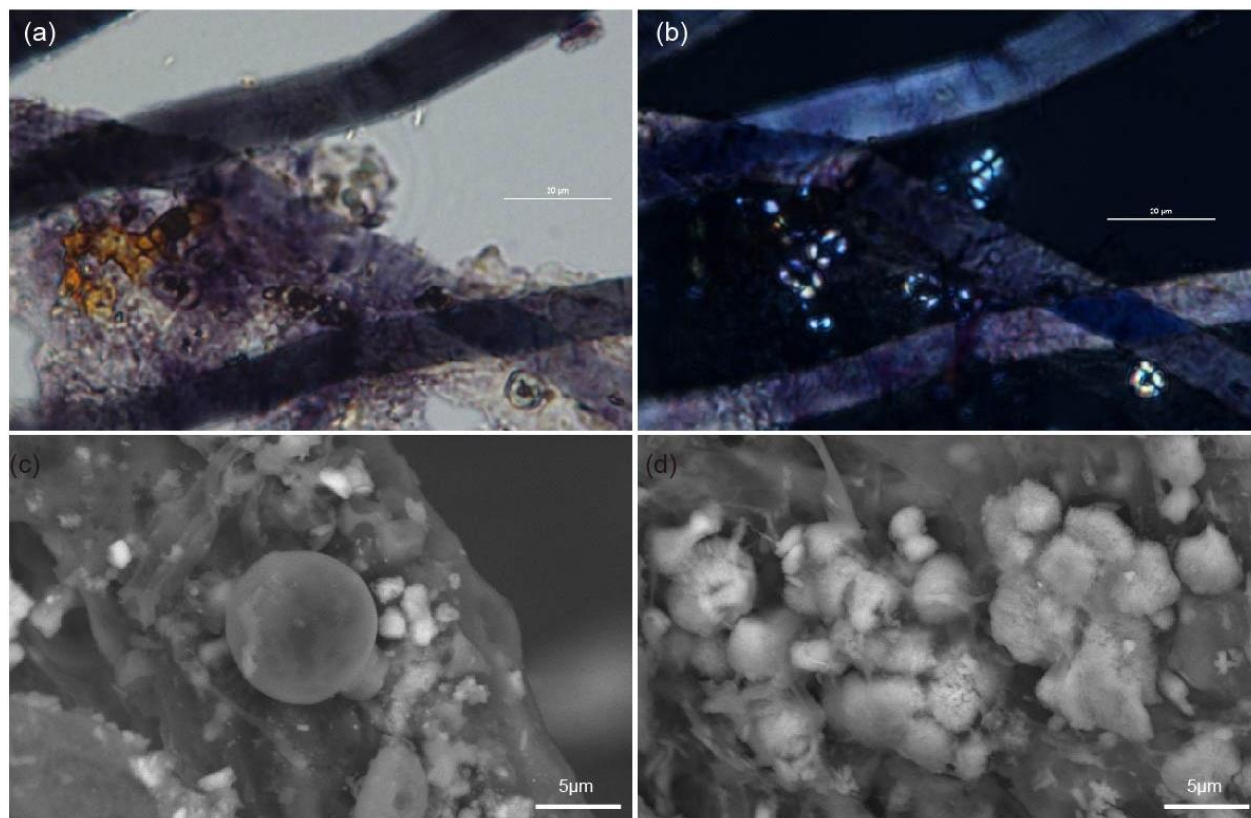




**Figure 5. Py-GC×GC/MS chromatogram of the total ion current of the ancient Tibetan paper sample with labelled casein peaks reported in Table 2.**

### 3.5 the filler and sizing agent

It is interesting to observe starch grains through microscopic observation. Starch granules have a semi-crystalline structure chemically composed of polysaccharides amylose and amylopectin. As is well known, the starch granules exhibit the cross extinction phenomenon especially under polarizing conditions of light. A large number of starch granules can be observed as can be seen from Fig.6. As previously well documented, the starch granule's physicochemical properties as well as its structure will be affected when exposed to heating [40]. The gelatinization process that underwent a series of irreversible structural and morphological changes with concurrent loss of birefringence that can be observed under cross-polarized microscopy [41]. The observation of the starch within the paper fragment were mostly small in diameter (less than 10  $\mu\text{m}$ ) and in its native and unaltered form while some larger swollen broken granules can be observed (*e.g.*, (d) in Fig.S5). Discrete spherical starch granules and paste agglomerations can also be observed through SEM observation which were mainly composed of C and O that corresponds well to the presence of starch grains (Fig.6c, (a)-(e) in Fig.S5, Fig.S6 in Supplementary Information) then confirming the previous microscopic observation under polarized light and resembling previously documented cereal crops starch microstructures [42]. These features were also observed within other sized ancient paper fragments (*e.g.*, the Dunhuang manuscripts [43] and Endere Tibetan ancient paper fragments [13]).



**Figure.6 the observation of unaltered starch grains under transmitted light (a) and under polarized light (b) as well as by SEM (c). (d) is the observation of calcium carbonate particles by SEM. More SEM observation images as well as the EDS analysis results are provided in Fig.S5, Fig.S6 and Table S1 in Supplementary Information.**

The detection of starch grains and paste revealed that starch was mainly used as a sizing agent (underwent gelatinization process), exceeding the role of some kind of filler when forming agglomerations (the unaltered granules). A sizing agent recipe combining animal glue (not detected in Py-GCxGC/MS and Proteomic analysis) and alum ( $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ) as precipitant, observed in contemporary central China, was less likely used in the treatment of this ancient Tibetan fragment. As demonstrated by the EDS results in which aluminum (Al) and sulfur (S) were none detected or in trace level (except point 28 and 36 in Fig.S6 and Table S1, Supplementary Information, while the coexistence of silicium (Si) may indicate environmental sources of these elements).

Besides the unaltered starch grain, the possible presence of inorganic fillers was examined by conducting energy-dispersive X-ray spectroscopy (EDS) elemental analysis. Large content and aggregation of particles has been observed on the surface as well as inside the paper fragment under SEM observation ((f)-(i) in Fig.S5, Supplementary Information) and the elemental analysis revealed these particles were mainly composed of carbon (C), oxygen (O) and calcium (Ca) (Fig.S6, Supplementary Information) which correspond to calcium carbonate ( $\text{CaCO}_3$ ), a common filler for paper treatment. Thus, the feature for the fillers applied to the paper fragment

was a combination of organic material (unaltered starch grains) and inorganic material (calcium carbonate), a treatment which serves to make the paper denser, stronger and whiter.

### 3.6 Proteomic analysis

Proteomic analysis of the fragmented paper resulted in the identification of 9 proteins, whose detailed information were displayed in Table S2, Supplementary Information. At least one unique peptide was identified in each protein. All these peptides were further BLAST searched in NCBI to confirm their accurate origins. Two milk proteins from *Bovinae*, alpha S1 casein and beta casein, have been identified in this sample. It indicated that bovine milk was added into the paper, probably function as an organic binder [44, 45]. Seven proteins from *Triticeae* were also detected, mostly originating from seeds. Alpha-gliadin and gamma-gliadin were the major storage proteins in endosperm, whose content influence the quality of wheat flour [46]. Gliadins have been used as typical “fingerprinting” biochemical markers for the screening, classification and identification of wheat cultivars [47, 48]. However, the identified peptides in this sample could only assign these proteins to *Triticeae* or *Triticinae* (Table S1). Another group of plant proteins, alpha-amylase inhibitor CM2 and alpha-amylase/trypsin inhibitor CM3 have more specific peptides, which could be attributed to *Triticum spp.* Fig.7 shows the MS/MS spectra of two specific peptides EmQWDFVR from *Triticum spp.* and GSPVSTEPGNTPR from common wheat (*Triticum aestivum*). The CM2 and CM3 types of  $\alpha$ -amylase inhibitor were reported to be mainly expressed in seeds and have a role of controlling the chromatin activity [49]. Alpha-amylase/trypsin inhibitor CM3 has previously been identified in the mortars of ancient Chinese wooden buildings, indicating wheat flour (*Triticum aestivum*) as organic additives [50]. Similarly, in this study, morphological observation suggested the use of starch from cereal crops as sizing agent while proteomics confirmed its specific species origins. Although wheat flour is mainly composed of polysaccharides, its protein content related to the production or degradation of polysaccharide chains can be used as indicators of plant starch origins [51].

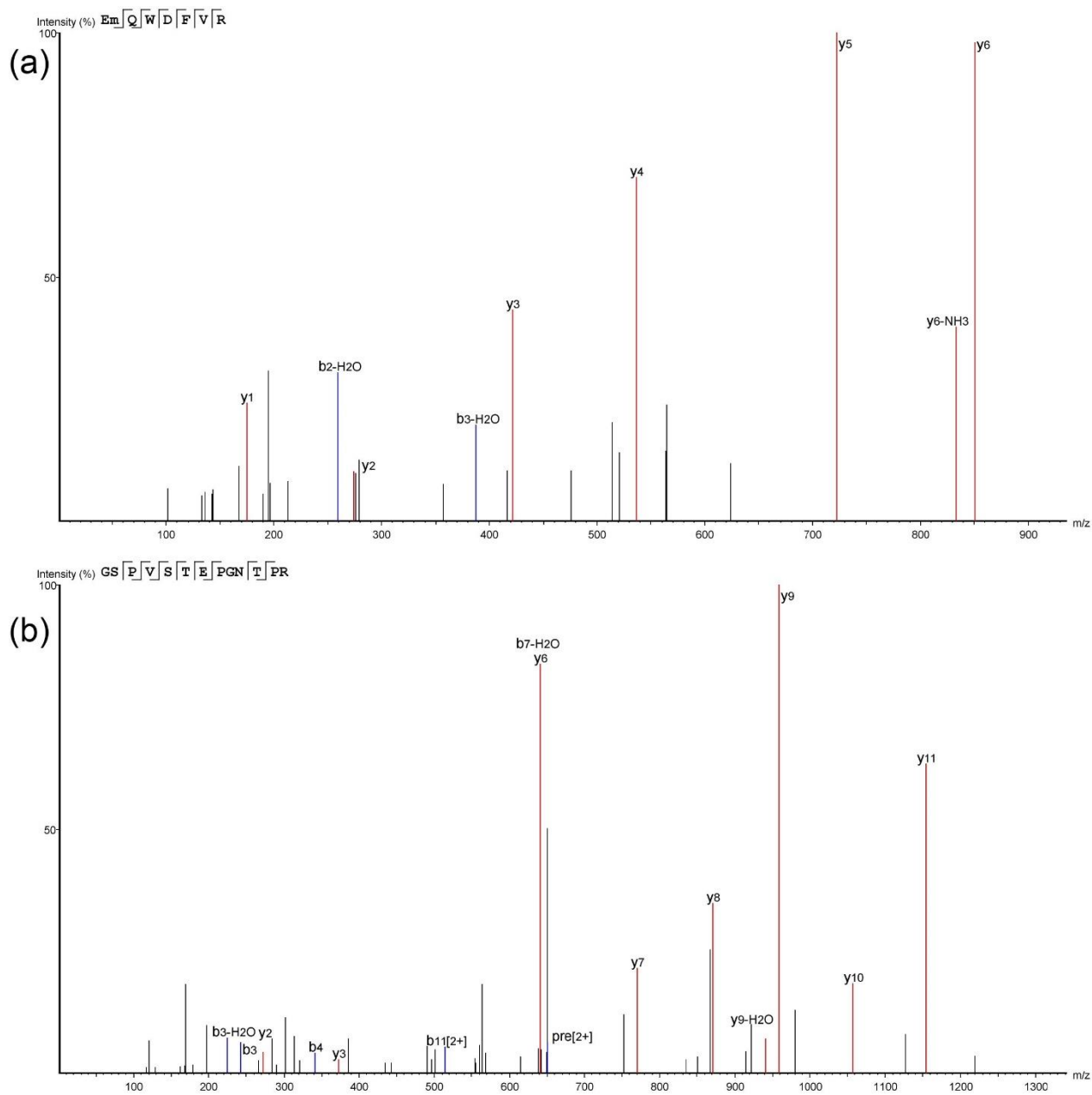


Fig.7 MS/MS spectra of two specific peptides. (a) EmQWDFVR from *Triticum spp.*; (b) GSPVSTEPGNTPR from common wheat (*Triticum aestivum*).

## 4. Discussions

### 4.1 the use of *Stellera chamaejasme* fiberfor Tibetan papers

The origin of the Tibetan hand papermaking is currently unknown and no details can be recovered from historical records. The historical origin of Tibetan papermaking might be diverse and difficult to determine in lack of detailed written descriptions [1]. It is viewed by some historical scholars that the allied marriage between Tang Dynasty and Tobu marked with the Wen Cheng princess entering Tubo and marrying Srongtsen Gampo (641 AD), started a stream for the

Central Plain Culture entering Tubo. The paper making technique as well as the craftsmen associated knowledge may have been developed along around this period. Due to the difference in the availability of raw plant resources between central China and Tibet Plateau, the papermaking craftsmen may have selected and explored local plant fibers that were suitable for papermaking. Thus, the locally adopted Tibetan papermaking will be revealed different from that of central China.

In fact, until now the two main sources of old Tibetan manuscripts that have been systematically studied were all out of the Tibet Plateau: as reported above, one is the Stein's discovery of Endere paper fragments [9, 10] and the other is Dunhuang manuscripts (Fig.1) [1]. The discovery of 27 pieces of eighth century Tibetan paper fragments in Endere out of Tibet Plateau had highlighted the topic of Tibetan paper origin and making method. According to the microscopic examination of prof. J. Wiesner, the manuscript fragment is made of pure and well-macerated raw fibers of a *Thymelaeacea* family plant, like *Daphne spp* and *Edgeworthia spp* [12, 13]. Wiesner in the 1900s had discovered the materiality of Tibetan paper fragment from *Thymelaeacea* family plant widely distributed within the Himalayan regions which supports the import of these fragments from Tibetan to Endere (*Thymelaeacea* family plants were not locally grown in Endere). It can be inferred from the results that by the eighth century, the use of *Thymelaeacea* family plant fibers for the making of Tibetan papers had been explored and correspondingly, the papers had traveled to the places where the power of Tibetan reaches.

The Chinese documents in the 'library' cave of Dunhuang mostly have colophons for dating while the Tibetan fragments don't have clear dating information. As a consequence, these fragments can only be approximately attributed from between the late eighth century and early eleventh century. In the analysis of 63 samples (within 50 manuscripts) of Dunhuang manuscripts from Stein Collection [1], three types can be classified: (I) manuscripts composed of ramie and hemp; (II) manuscripts composed of *Daphne spp* or *Edgeworthia spp.*; (III) manuscripts composed of paper mulberry. The features of the (I) type were interpreted as locally made in Dunhuang and evidence showed that (II) and (III) were produce in Tibet especially for type (II) manuscripts [1]. The analysis of the Endere and Dunhuang Tibetan manuscript supported that Tibetan papermaking in treatment of *Thymelaeacea* family plant fibers could probably mature around the 8~9<sup>th</sup> century.

*Stellera chamaejasme* is a stout perennial species that has been widely acknowledged by Tibetans to be poisonous for animals and can be used as medicines after treatment. Growing even at altitudes above 5000 m, *Stellera* sp. permits production of paper in the highest places where other paper plant species doesn't commonly grow. Ecologists have suggested that the vegetation on the Tibet Plateau has been influenced by human activity for several centuries especially from Tang Dynasty onward with a high grazing intensity and a corresponding rapid population increase suggested by the dramatically increase of *Stellera* in the pollen record, an indicator for the grassland degradation [52]. This environmental observation was in accord with historical descriptions that when papermakers came with Wen Cheng princess to Tubo, the common

ingredient of papermaking was inaccessible and alternatively they adapted the abundant and well-distributed *Stellera* as the raw papermaking materials which is suitable for Tibetan's hard-tipped pen writing.

It has been noted that the Tibetan *Stellera chamaejasme*, as unique raw material for papermaking, has not been observed in current examination of Endere and Dunhuang fragments [1]. *Thymelaeaceae* family plant fibres (specifically *Daphne* or *Edgeworthia* sp.) are reported as scarcely identified in ancient Tibetan manuscripts [53]. The early description of *Stellera chamaejasme* in the Tibetan papermaking could be traced back to 1918 by British consular officer Eric Teichman: "I came across some Tibetans digging up the root of a kind of bush from which Tibetan paper is made. This paper is used all over Tibet". *Stellera* was regarded as high-quality paper and has been used for special purpose. Missionary Duncan also recorded the use of *Stellera chamaejasme* root for paper making in Dege County during his stay between 1921-1932 [36]. However, Tibetan papermakers likely have preferred to use *Daphne*/*Wikstroemia*/*Edgeworthia* than *Stellera chamaejasme* when possible since this latter plant is known to necessitate a longer time for processing, in particular, its roots are difficult to be extracted from the soil, and the whole papermaking process requires an extra step in processing [36]. Consequently, it explains why *Stellera chamaejasme* is reported to be used in a few printing houses and papermaking centers.

The earliest old fragment that contained the *Stellera* spp. fiber dated to the 11<sup>th</sup> century were reported in Central Tibet [54, 55]. Our analyzed sample was from the same county (Samye Monastery) as the one reported by Professor Helman-Wazny in one of her publications [56]. Considering the characterization of *Stellera chamaejasme* both by microscopic analysis of the fibers and Py-GC/MS analysis of ancient Tibetan paper samples, it can be reached that between the 11~13<sup>th</sup> century, *Stellera chamaejasme* has been very likely used within Tibet for the process of hand papermaking.

#### 4.2 a new perspective on the way starch sizing agent prepared

Apart from the species of plant used in papermaking, the use of the starch as a sizing agent is another point of great interest. The unusual phenomenon how starch grain was used was firstly observed by Professor J. Wiesner: it is not by 'sizing' with a glue of starch (gelatinization), but by heavily 'loading' the paper with unaltered (ungelatinised) starch of rice flour [12, 13]. Important as it is, it seems this phenomenon of starch use has scarcely been reported and discussed later on. If we look closely at the results gained from the analysis of the Samye Monastery paper samples, it appeared that the Tibetan fragment was 'loaded' with unaltered (ungelatinized) starch. However, in the common way of using starch as adhesive glue, it was usually heated in a solution that caused the gelatinization of the starch grain to be viscous starch paste, a method that has been widely used in paper treatment since 4<sup>th</sup> century until nowadays.

In fact, one critical aspect has not been pointed out for the observation of Prof. J. Wiesner: if the starch was not unaltered (ungelatinized), it cannot function as adhesive and the physical ‘loading’ method cannot guarantee a strong bond between the cellulose fibers and the starch granule which may predict to fall off during usage. And also, this ungelatinized ‘loading’ cannot add the impermeability of the cellulose fiber to the liquid ink with a low pasting behavior. Although Prof. J. Wiesner pointed out the possibility of using starch milk (‘Stärkemilch’), that is, with a liquid in which starch was so abundantly suspended that it had the appearance and consistency of a somewhat thickened milk while the essence of the liquid cannot be specified at that time [13].

In our analysis, we also observed similar phenomenon with the presence of numerous ungelatinized starch granule through microscopic observation (Fig.6). Nevertheless, the fragment surface seems to be plain and tight, the peeled off of the starch grain has not been observed, which means the cellulose fibers and the ungelatinized starch granule has been well bonded. It is then difficult to explain this fact from the results of Py-GC/MS and SEM-EDS analysis since the use of alum-gelatin sizing as binders was not observed.

The detection of casein through proteomics analysis, however, provides some clue for the explanation of the phenomenon of sizing considering that milk protein can influence the starch gelatinization process. Casein is the main protein within the milk of mammals which, in our case, refines to the bovine milk (in considering its origin in Tibet Plateau, it might be inferred for the yak native to Tibet Plateau). As for starch, gelatinization occurs when starch is heated in a water solution condition, the amylopectin double helices dissociate, accompanying the loss of crystallinity while the granules swell with the imbibed water. The breaking down of the crystalline structure release amylose and amylopectin that increase the viscosity of the system, forming a starch paste which can be used as a sizing agent in paper sizing and mounting. It has been proposed that the starch pasting behavior are ruled by two factors: the amylose content of the starch and the restriction of swelling by a layer of lipid and protein on the surface of the granule [57]. Although all starch granules (in the presence of water) swell when heated, the granule swelling behaviors in rate and extent can be markedly influenced by surface proteins and lipids [58]. In a starch and milk protein co-existing system, the milk protein would be adsorbed onto the starch granules that reduce the swell by absorbing water and in the process alpha-casein adsorption is found preferential and in higher amounts than beta-casein [59]. As demonstrated through polarized light microscopic observation, at lower temperature (25 °C), 5% weight waxy maize starch showed intact granules with a Maltese cross pattern of ungelatinized starch; while when heated to 75 °C (a temperature for which the gelatinization endotherm in Differential Scanning Calorimetry is close to completion), only a few unaltered granules can be observed, comparatively, far more intact granules were remained in the mixtures when the same starch mixed with 5% weight alpha-caseinate or beta-caseinate (and only when the temperature was raised to 80 °C, the disappearance of the intact granule became the same with or without caseinate) [60]. The observation through confocal laser scanning microscopy with fluorescent labelling by the same authors also revealed that the swelling of starch is already evident at



temperature of 70 °C (without addition of proteins) accompanied by the onset of increase in viscosity during pasting. Under the same conditions, in the mixtures with alpha-casein or beta-casein, far less swelling has been observed while some of the granules remained to be completely ungelatinized accompanied by a later onset of increase in viscosity. The actual dairy protein-starch interaction is complex but it can be rationalized in the fact that caseinates can make gelatinized granules tougher and smaller during the gelatinization process [60, 61].

The observation of Prof. J. Wiesner for the heavily loaded paper with unaltered starch as well as the similar observation in our analysis of paper fragments from Samye Monastery could be reasonably explained: the ‘unaltered’ starch granule itself is actually not evidence for the use of (ungelatinized) starch from flour; on the contrary, the starch has been undergoing gelatinization process in the presence of dairy protein in a not high temperature (*e.g.*, less than 75°C); the presence of the milk protein makes more individual starch granule tougher resisting the swelling in the heating process while still, some starch granules broke off to increase the viscosity of the solution to be used as adhesive starch glue. By preparing the starch glue adding milk, a viscous solution can be made that can strengthen the starch-cellulose fiber bonding, improve the impermeability to modulate the permeation of liquid ink, but still, a number of intact starch granules can be observed through polarized microscopic observation. Noteworthy, milk casein has also been detected in the binders in ancient Tibetan murals, indicating its wide involvement in the manufacturing of ancient Tibetan writing and painting materials [62].

On a second matter, the detection of wheat (*Triticum* spp.) flour instead of rice (*Oryza sativa*, a source for starch mentioned by Stein and Wiesner in ancient Tibetan papers [12, 13]), could find an explanation in the suitability of wheat culture with the climate of Tibet Plateau. One can notice that rice is mainly and geographically cultivated in the southern plain regions of China while most parts of the Tibet Plateau are more suitable to cultivate drought-enduring and cold-resistant species such as wheat and barley. Thus, comparatively, wheat is easier accessible and more cost-effective to be used as starch origins for a sizing agent within Tibetan regions.

As an explanation, we suggest from the analytical results that in the making of the ancient Tibetan manuscript, milk was added in the preparation of the starch glue from wheat flour and applied to the fragments. These discovery makes the paper fragment under discussion so extraordinarily interesting and leads to reveal a hidden sizing recipe. Such a craft has never been revealed before and it stimulates further study on the sizing procedure and impermeability of these new ingredients and recipes. The possibility for the detected milk casein coming from contaminations during storage or usage was less possible since that would cause discrepancy in the <sup>14</sup>C radiocarbon dating with the paleography analysis which has not been observed in this study.

## 5. Conclusion



The study presents the first multidisciplinary investigation of a set of ancient Tibetan paper fragments dated from the 12<sup>th</sup>~13<sup>th</sup> century AD with unequivocal scientific evidence for the use of *Stellera chamaejasme* L., a native plant of the Tibetan Plateau, as fiber materials, and the presence of a sizing agent made from wheat flour (*Triticum spp*) and proteins sourcing from bovine milk. *Stellera chamaejasme* is known to be used in Tibetan manuscripts and usually related to a local origin, while other plants already observed in Tibetan manuscripts like hemp, *Daphne* or *Edgeworthia* are more correlated with Tibetan dynasty central Asia history. The milk-starch sizing agent was for the first time revealed in Tibetan hand papermaking history. The use of wheat flour (*Triticum spp*) can be rationalized by the fact that it is easy to access in the region of paper origin due to its wide cultivation around the Tibet Plateau. Milk proteins in particular casein attributed to *Bovinae* are more likely originating from native yak. The fillers within the fragment composed of organic material (unaltered starch grains) and inorganic material (calcium carbonate) that made the denser, stronger and whiter.

These characterizations permit to propose first pieces of evidence on a sizing recipe specific to the ancient Tibetan paper craft since little information was known about the early Tibetan papermaking crafts insofar. Combination of different analytical methods microscopy, SEM, Py-GC/MS, Py-GCxGC/MS confirm the identity of constituents involved in the sizing recipe, while proteomics permitted to refine the presence of these firstly detected constituents. From our experimental results, it was proposed that the addition of milk proteins influences the starch gelatinization process, which is justified by the observation of numerous smaller intact starch granules within the gelatinized starch paste coated in the analyzed ancient paper fragments. These experimental results provide the first evidence that bovine milk had been involved in Tibetan papermaking. The new analytical strategy (Py-GC/MS, Py-GCxGC/MS, proteomics) proposed here with its innovative approaches permits to not only study the tangible materials of ancient papermaking but also to reveal the underlying intangible intelligence and cultural communication of ancient Tibetan craftsmen, gaining new insights into the early development of hand papermaking.

## Acknowledgements

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## REFERENCE

1. Helman-Wazny, A. and S. Van Schaik, Witnesses for Tibetan craftsmanship: bringing together paper analysis, palaeography and codicology in the examination of the earliest Tibetan manuscripts. *Archaeometry*, 2013. 55(4): p. 707-741.
2. Helman-Wazny, A., Historical, Art-Historical, and natural science approaches to dating Tibetan books- possibilities and limitations. in *Tibetan Studies. An Anthology [PIATS 2006: Proceedings of the Eleventh Seminar of the International Association for Tibetan Studies]*, Saadet Arslan and Peter Schwieger Eds., Königswinter 2006, p 279-295.
3. Shengzhang, H., The time and route of the introduction of Chinese paper and papermaking method into the Indian subcontinent. *Historical Research*, 1980(01): p. 113-133. (*in Chinese*)
4. Jianchang, F., Papermaking industry in ancient Tibet. *Journal of Chinese Historical Geography*, 1994(04): p. 133-145. (*in Chinese*)
5. Rinchen, S. and G.C. Dorje, An investigation of Tibetan traditional papermaking technology. *China Tibetology*, 2010(02): p. 72-83+2.
6. Shi, J.L. and T. Li, Technical investigation of 15th and 19th century Chinese paper currencies: Fiber use and pigment identification. *Journal of Raman Spectroscopy*, 2013. 44(6): p. 892-898.
7. Li, X., Study on the Tibetan Traditional Papermaking in Sichuan's Dege County and Tibet's Nimu County *The Chinese Journal for the History of Science and Technology*, 2007(02): p. 155-164. (*in Chinese*)
8. Bogle, G. and T. Manning, *Narratives of the mission of George Bogle to Tibet: and of the journey of Thomas Manning to Lhasa*. 1879: Trübner and Co.
9. Stein, A., Preliminary report on a journey of archaeological and topographical exploration in Chinese Turkestan. 1901: Eyre and Spottiswoode.
10. Stein, A., Sand-buried ruins of Khotan: personal narrative of a journey of archaeological and geographical exploration in Chinese Turkestan. 1904: Hurst and Blackett.
11. Barnett, L., Preliminary notice of the Tibetan manuscripts in the Stein collection. *The Journal of the Royal Asiatic Society of Great Britain and Ireland*, 1903: p. 109-114.
12. Stein, A., *Ancient Khotan: Detailed report of archaeological explorations in Chinese Turkestan*. Vol. 1. 1907: Clarendon Press.
13. Wiesner, D.J., *Ein neuer Beitrag zur Geschichte des Papieres*, von J. Wiesner. 1904: C. Gerold's Sohn.
14. van Schaik, S., A. Helman-Wazny, and R. Nöller, Writing, painting and sketching at Dunhuang: assessing the materiality and function of early Tibetan manuscripts and ritual items. *Journal of Archaeological Science*, 2015. 53: p. 110-132.
15. Almogi, O., et al., Inks, pigments, paper: in quest of unveiling the history of the production of a tibetan buddhist manuscript collection from the tibetan-nepalese borderlands. *Journal of the International Association of Buddhist Studies*, 2013: p. 93-118.
16. Gong, Y., et al., Analytical study of the special crafts used in Ancient Tripitaka. *Journal of Cultural Heritage*, 2015. 16(6): p. 928-933.

17. Salvemini, F., et al., An in situ non-invasive study of two Tibetan manuscripts from the Asian Collection of the Museum of Natural History in Florence. *Archaeological and Anthropological Sciences*, 2018. 10(8): p. 1881-1901.
18. Liu, Y., et al., Identification of Proteinaceous Binders in Ancient Tripitaka by the Use of an Enzyme-linked Immunosorbent Assay. *Analytical Sciences*, 2016. 32(7): p. 735-740.
19. Avataneo, C. and M. Sablier, New criteria for the characterization of traditional East Asian papers. *Environmental Science and Pollution Research*, 2017. 24(3): p. 2166-2181.
20. Han, B., et al., Identification of traditional East Asian handmade papers through the multivariate data analysis of pyrolysis-GC/MS data. *Analyst*, 2019. 144(4): p. 1230-1244.
21. Han, B., G. Daheur, and M. Sablier, Py-GC<sub>x</sub>GC/MS in cultural heritage studies: an illustration through analytical characterization of traditional East Asian handmade papers. *Journal of analytical and applied pyrolysis*, 2016. 122: p. 458-467.
22. Han, B., et al., Analytical characterization of East Asian handmade papers: a combined approach using Py-GC<sub>x</sub>GC/MS and multivariate analysis. *Journal of analytical and applied pyrolysis*, 2017. 127: p. 150-158.
23. Han, B., S. Lob, and M. Sablier, Benefit of the Use of GC<sub>x</sub>GC/MS Profiles for 1D GC/MS Data Treatment Illustrated by the Analysis of Pyrolysis Products from East Asian Handmade Papers. *Journal of The American Society for Mass Spectrometry*, 2018. 29(8): p. 1582-1593.
24. Helman-Ważny, A., L. Kriakina, and A. Zorin, The First Tibetan Leaves Acquired by the St. Petersburg Academy of Sciences: Conservation Issues, Contents and Paper Analysis. *Written Monuments of the Orient*, 2015(2): p. 61-76.
25. Helman-Ważny, A., *The archaeology of Tibetan books*. 2014: Brill.
26. Drashi, B.L., A Study of the Frescoes of Jangchub Semkye Hall in Samye Monastery. *China Tibetology*, 2006(01): p. 59-67.
27. Reimer, P.J., et al., IntCal13 and Marine13 radiocarbon age calibration curves 0–50,000 years cal BP. *Radiocarbon*, 2013. 55(4): p. 1869-1887.
28. Ramsey, C.B., Methods for summarizing radiocarbon datasets. *Radiocarbon*, 2017. 59(6): p. 1809-1833.
29. Warinner, C., et al., Pathogens and host immunity in the ancient human oral cavity. *Nature Genetics*, 2014. 46(4): p. 336-344.
30. Warinner, C., et al., Direct evidence of milk consumption from ancient human dental calculus. *Scientific Reports*, 2014. 4: p. 7104.
31. Hendy, J., et al., Proteomic evidence of dietary sources in ancient dental calculus. *Proceedings of the Royal Society B: Biological Sciences*, 2018. 285(1883).
32. van Schaik, S., Dating early Tibetan manuscripts: a paleographical method. *Scribes, Texts and Rituals in Early Tibet and Dunhuang*, 2013: p. 119-135.
33. Oda, H., et al., Radiocarbon dating of ancient Japanese document and calligraphy. *Japan Geoscience Union Meeting 2015*.
34. Mai, H., et al., Investigating the materials and manufacture of Jinzi: The lining of Futou (Chinese traditional male headwear) from the Astana Cemeteries, Xinjiang, China. *Journal of Cultural Heritage*, 2017. 27: p. 116-124.

35. Jawhari, T., A. Roid, and J. Casado, Raman spectroscopic characterization of some commercially available carbon black materials. *Carbon*, 1995. 33(11): p. 1561-1565.
36. Boesi, A., Paper Plants in the Tibetan World: A Preliminary Study, in *Tibetan Printing: Comparison, Continuities, and Change*. 2016, BRILL. p. 501-531.
37. Cersoy, S., et al., Pyrolysis comprehensive gas chromatography and mass spectrometry: a new tool to assess the purity of ancient collagen prior to radiocarbon dating. *Analytica chimica acta*, 2018. 1041: p. 131-145.
38. Chiavari, G., et al., Analysis of Proteinaceous Binders by in-situ Pyrolysis and Silylation. *Chromatographia*, 2003, 57 (9-10), 645-648.
39. Chiavari, G. and S. Prati, Analytical pyrolysis as diagnostic tool in the investigation of works of art. *Chromatographia*, 2003. 58(9-10): p. 543-554.
40. Crowther, A., The differential survival of native starch during cooking and implications for archaeological analyses: a review. *Archaeological and Anthropological Sciences*, 2012. 4(3): p. 221-235.
41. Dai, J., et al., Changes in wheat starch grains using different cooking methods: Insights into ancient food processing techniques. *Chin Sci Bull*, 2013. 58(Suppl I): p. 82-89.
42. Valamoti, S.-M., et al., Prehistoric cereal foods from Greece and Bulgaria: investigation of starch microstructure in experimental and archaeological charred remains. *Vegetation History and Archaeobotany*, 2008. 17(1): p. 265-276.
43. Shouji, S. and Y. Okada, Analysis of Particles on the Paper of Dunhuang Documents. *Archeology and Natural Sciences: Journal of the Japanese Society of Cultural Properties Science*, 2017(72): p. 63-75. (*in Japanese*)
44. Leo, G., et al., Proteomic strategies for the identification of proteinaceous binders in paintings. *Analytical and Bioanalytical Chemistry*, 2009. 395(7): p. 2269-2280.
45. Chambery, A., et al., Improved procedure for protein binder analysis in mural painting by LC-ESI/Qq-TOF mass spectrometry: detection of different milk species by casein proteotypic peptides. *Analytical and Bioanalytical Chemistry*, 2009. 395(7): p. 2281-2291.
46. Bonilla, J.C., M.Y. Erturk, and J.L. Kokini, Understanding the role of gluten subunits (LMW, HMW glutenins and gliadin) in the networking behavior of a weak soft wheat dough and a strong semolina wheat flour dough and the relationship with linear and non-linear rheology. *Food Hydrocolloids*, 2020. 108: p. 106002.
47. Piergiovanni, A.R., Estimating Gliadin and Albumin Variation at Intra - and Interaccession Level in USDA Oriental Wheat (*Triticum turgidum* L. subsp. *turanicum* (Jakubz.)(A. Löve & D. Löve) Collection Using Capillary Zone Electrophoresis. *Cereal chemistry*, 2009. 86(1): p. 37-43.
48. Schober, T.J. and M. Kuhn, Capillary zone electrophoresis for gliadin separation: applications in a spelt breeding program. *European Food Research and Technology*, 2003. 217(4): p. 350-359.
49. Mancinelli, L., et al., The CM2 and CM3 types of  $\alpha$ -amylase inhibitor are associated with *Triticum aestivum* seed chromatin. *Plant Physiology and Biochemistry*, 2003. 41(8): p. 705-710.

50. Rao, H., et al., Proteomic identification of organic additives in the mortars of ancient Chinese wooden buildings. *Analytical Methods*. 2015. 7(1): p. 143-149.
51. Kuckova, S., et al., Characterization of proteins in cultural heritage using MALDI-TOF and LC-MS/MS mass spectrometric techniques. *Physical Sciences Reviews*, 2018. 4(5).
52. Huang, X.-z., et al., Early human impacts on vegetation on the northeastern Qinghai-Tibetan Plateau during the middle to late Holocene. *Progress in Physical Geography*, 2017. 41(3): p. 286-301.
53. Helman-Wazny A., Local papermaking, paper trade and book-culture promotion in Inner Asia, in *IPH Congress book 17/2008* (ed. J.-E. Levlin), 177-90, Stockholm. 2009.
54. Wangdu, P., Paper, Patronage and Production of Books: Remarks on an 11th Century Manuscript from Central Tibet, in *Tibetan Printing: Comparison, Continuities, and Change*. 2016, BRILL. p. 555-559.
55. Wangdu, P. Ke Ru Lha Khang: Cultural Preservation And Interdisciplinary Research In Central Tibet. in *Proceedings of the Tenth Seminar of the IATS*, 2003. Volume 7: Text, Image and Song in Transdisciplinary Dialogue. 2007. BRILL.
56. Helman-Ważny, A., The Choice of Materials in Early Tibetan Printed Books, in *Tibetan Printing: Comparison, Continuities, and Change*. 2016, BRILL. p. 532-554.
57. Debet, M.R. and M.J. Gidley, Why Do Gelatinized Starch Granules Not Dissolve Completely? Roles for Amylose, Protein, and Lipid in Granule “Ghost” Integrity. *Journal of Agricultural and Food Chemistry*. 2007. 55(12): p. 4752-4760.
58. Debet, M.R. and M.J. Gidley, Three classes of starch granule swelling: Influence of surface proteins and lipids. *Carbohydrate Polymers*. 2006. 64(3): p. 452-465.
59. Noisuwan, A., et al., Adsorption of milk proteins onto rice starch granules. *Carbohydrate Polymers*. 2011. 84(1): p. 247-254.
60. Kett A.p. et al. Influence of milk proteins on the pasting behaviour and microstructural characteristics of waxy maize starch. *Food Hydrocolloids*. 2013, 30(2): 661-671.
61. Kumar, L., et al., Rheological, Pasting and Microstructural Studies of Dairy Protein-Starch Interactions and their Application in Extrusion-Based Products: A Review. *Starch-Stärke*, 2017. 69(1-2): 1600273.
62. Wu, M., et al., Immunological methods for the detection of binders in Ancient Tibetan Murals. *Microscopy and Microanalysis*, 2019. 25(3): p. 822-829.