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

3  
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16  
17 **ABSTRACT**

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

38  
39 Keyword: Tibetan paper, *Stellera Chamaejasme* fiber, sizing technique, starch-milk sizing, casein,  
40 Py-GC/MS, proteomics

## 41 1. Introduction

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

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

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

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

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

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

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



109

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

112

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

121

## 122 **2. Materials and methods**

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

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

### 133 **2.1 Sample collection**

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



141  
142 **Figure.2 the paper fragments preserved in the Samye Monastery.**

143 **2.2 <sup>14</sup>C radiocarbon dating**

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

154 **2.3 Microscopic observation**

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

165 **2.4 Py-GC/MS and Py-GCxGC/MS Analysis**

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

169 (Shimadzu, Champs-sur-Marne, France). The sample was placed in a stainless-steel sample cup.  
170 The paper samples were weighted with an XP2U Ultra Micro Balance (Mettler Toledo, Viroflay,  
171 France). Typical quantities of reference paper used in this work for the development of the  
172 analytical method ranged from 80 to 110  $\mu\text{g}$  weighted with the microbalance. For the balance's  
173 capacity used (2.1g), readability and repeatability of the microbalance were 0.0001 and 0.0002  
174 mg, respectively. The sample cup was placed on top of the pyrolyzer at near ambient temperature.  
175 The sample cup was introduced into the furnace at 500  $^{\circ}\text{C}$ , and then the temperature program of  
176 the gas chromatograph oven was started. The Py-GC interface was held at 320  $^{\circ}\text{C}$ .  
177 Chromatographic separation was carried out on an Agilent DB5-ms 5% phenyl-95% dimethyl  
178 polysiloxane fused silica capillary column (30 m length, 0.25 mm inner diameter and coated with  
179 a 0.25  $\mu\text{m}$  film thickness). The oven temperature was initially held 3 min at 40  $^{\circ}\text{C}$ , and then  
180 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  
181 was 70 min. The helium carrier gas Alphagaz 1 (Air Liquide, France) was used in the linear  
182 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  
183 total flow). The mass spectrometer was operated at 5000 u.s $^{-1}$ , with a scan range from 42 to 500  
184 u, using electron ionization at 70 eV. The interface was kept at 300  $^{\circ}\text{C}$  and the MS source at 200  
185  $^{\circ}\text{C}$ . The mass spectra of the pyrolysis products were recorded using the average spectrum +  
186 average and subtract spectrum modes in the Shimadzu GCMSsolution software. Identifications  
187 were achieved on the basis of EI mass spectra by interpretation of the main fragmentations [22]  
188 and using the NIST MS library (2011).

189 Py-GCxGC/MS analysis was conducted with a Shimadzu QP 2010 Ultra mass spectrometer  
190 (Shimadzu, Champs-sur-Marne, France) equipped with a two-stage thermal modulator ZX 2  
191 (Zoex, Houston, USA). Pyrolysis was performed using a vertical micro-furnace-type pyrolyzer  
192 PY-3030D (Frontier Lab, Fukushima, Japan) directly connected to the injection port of the gas  
193 chromatograph. The sample cups and the paper samples were weighted similarly than during Py-  
194 GC/MS experiments. A sample of 70  $\mu\text{g}$  was weighted here. The sample cup was placed on top  
195 of the pyrolyzer at near ambient temperature. The sample cup was introduced into the furnace at  
196 500  $^{\circ}\text{C}$  and the temperature program of the gas chromatograph oven was started. The pyrolyzer  
197 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  
198 thickness, Macherey-Nagel, Hoerdt, France) was used as first dimension column and a Zebron  
199 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  
200 a second-dimension column and for the loop modulator system. The separation was carried out at  
201 an initial constant pressure of 300 kPa in a constant pressure mode (which resulted in a flow rate  
202 varying from 1.0 mL/min to 0.4 mL/min during the acquisition), and using Helium Alphagaz 2 as  
203 carrier gas (Air Liquide, Bagnaux, France). The ZX 2 two-stage thermal modulator employs a  
204 closed cycle refrigerator/heat exchanger to produce a -90  $^{\circ}\text{C}$  cooled air jet regularly modulated  
205 with a pulsed hot air jet. The optimized modulation period for two-dimensional chromatogram  
206 collection was 9 s with a programmed hot pulse of 0.350 s. A multi-step temperature program  
207 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  
208 the acquisition. The oven temperature was initially held 1 min at 100  $^{\circ}\text{C}$ , and then ramped at 2  $^{\circ}\text{C}$



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

## 217 **2.5 Proteomics Analysis**

218 The fragmented sample was extracted with a filter-aided sample preparation protocol (FASP)  
219 modified from references [29-31]. Then extracted peptides were analyzed using tandem mass  
220 spectrometry (MS/MS). Peptides were loaded on a Thermo-Dionex Ultimate 3000 HPLC system  
221 interfaced with a Thermo Orbitrap Fusion Lumos mass spectrometer. The analytical column was  
222 Acclaim PepMapTM RSLC C18 capillary column (75 µm ID, 150 mm length; Thermo Fisher  
223 Scientific). Solvent composition at the two channels was 0.1% formic acid for channel A and  
224 0.1% formic acid in 100% acetonitrile for channel B. The peptides were separated with a  
225 135 min gradient elution at a flow rate 0.300 µL/min. Full-scan mass spectra were acquired in  
226 the Orbitrap with a m/z range of 300-1500 and a resolution of 120,000. Data-dependent MS/MS  
227 scans were acquired in an Orbitrap with a resolution of 15000 and 30% normalized collision  
228 energy in high energy collision induced dissociation (HCD) mode.

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

237

## 238 **3. Results**

### 239 **3.1 <sup>14</sup>C radiocarbon dating**

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

245 fragment found at Samye Monastery constitute part of Prajñāpāramitā Hṛdaya and was assumed  
246 to be dated from around the 13<sup>th</sup> century according to the paleography analysis. For confirmation  
247 of the origin, radiocarbon dating was conducted. The <sup>14</sup>C radiocarbon dating result indicates a  
248 calendar calibrated results of (1152-1260) cal AD (90.4% probability) and 1150-1219 cal AD  
249 (73.7% probability) by two independent radiocarbon dating laboratories (Fig.S1, Supplementary  
250 Information). The dating result showed agreement with the paleography analysis that the sample  
251 consists of a 12~13<sup>th</sup> century fragment. Although radiocarbon dating has proved to be an effective  
252 method to determine the written age of ancient Japanese documents [33], in the analysis of  
253 ancient Tibetan papers, most of the dating to date was conducted by paleography and codicology  
254 study while the first application of the radiocarbon dating method introduced in this study showed  
255 its efficiency and consistency in dating of ancient Tibetan papers in supplementary to  
256 paleography and codicology analysis.

### 257 **3.2 Microscopic observation**

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

267 In scientific analysis of paper artifacts, the starting point lies in the material origin itself which  
268 point to the plant fiber utilization. Paper fiber analysis is one of the most basic analysis method to  
269 obtain the paper components. It has been early observed that the local grown *Thymelaeaceae*  
270 family plants (*e.g.*, *Daphne* sp., *Edgeworthia* sp., *Stellera chamaejasme*) have been used for the  
271 manufacture of Tibetan paper from the evidence of field survey and ancient fragment analysis [8,  
272 12, 25]. To distinguish *Thymelaeaceae* family plant through fiber analysis is a big challenge in  
273 which the fiber analysis for clear-cut identification has been observed only in limited cases.  
274 However, it has to be remind that only a few species within *Thymelaeaceae* family has been  
275 historically utilized for papermaking [36]. Among them, the *Stellera chamaejasme* specie which  
276 grows locally in Tibet Plateau can be a characteristic component for exclusive identification of  
277 Tibetan manufacturing papers [25]. Furthermore, the fibers of *Stellera chamaejasme* were  
278 observed to be more distinguishable from the *Daphne* sp. and *Edgeworthia* sp. within the  
279 *Thymelaeaceae* family: the fiber presenting ribbon-like shape with the impression of transparency  
280 [25]. This kind of transparent ribbon-like shape fiber was observed within the analyzed fragment  
281 (Fig.S3, Supplementary Information) that gives hints for its fiber origin as *Stellera*  
282 *chamaejasme*'s fiber. Further analysis based on Py-GC/MS analysis confirmed this assertion as  
283 reported below (Part. 3.3).



284  
 285 **Figure.3** the state of the fragment under the microscopy: (a) the transverse scribal guidelines, (b) the ink diffusion state, (c)  
 286 and (d) the agglomeration and caking off the ink on the paper surface.

### 287 3.3 Py-GC/MS analysis

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

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

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

316 Table 1. Assignment of plant markers detected by Py-GC/MS in the ROI of the investigated  
 317 Tibetan papers, modern reference paper sample and ancient paper sample from the Samye  
 318 Monastery, with retention time ( $t_r$ ), main fragment ions, expected molecular weights, assigned  
 319 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

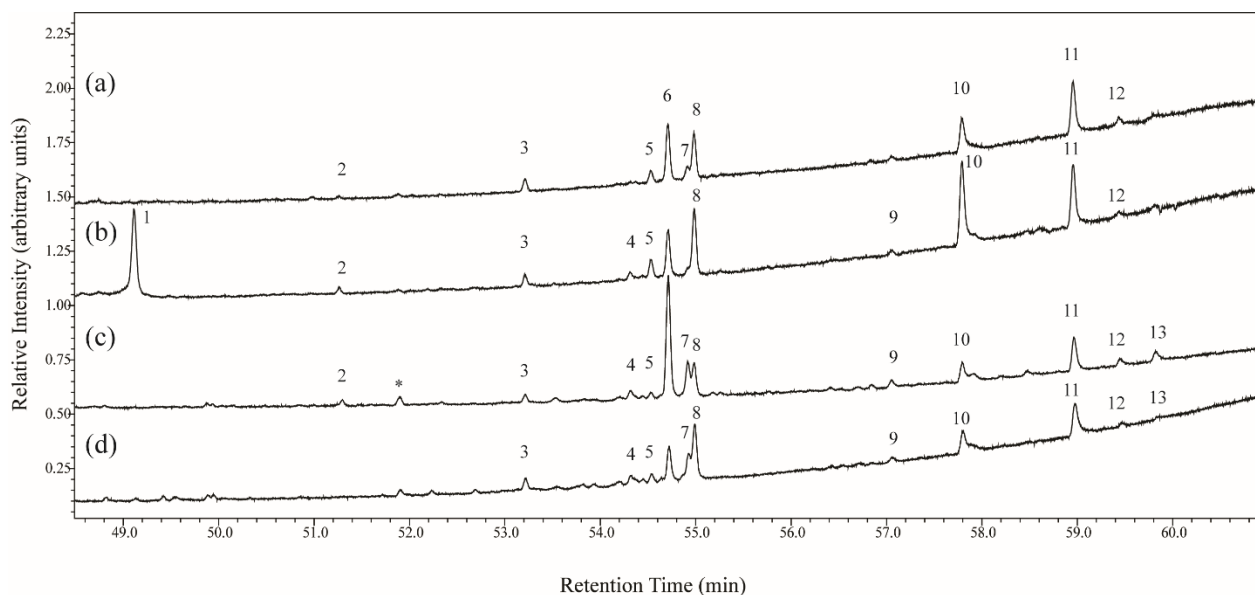
320 \* as previously reported [19] the fragment m/z 136 cast some doubt on this structure.

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

323 (#1, Table 1 and Fig.4) in the ancient Tibetan paper sample, and variations in the observed  
324 intensities of common markers with noticeable differences for some of them (#4, 7, 9, Table 1  
325 and Fig.4). On the other side, a good correlation appeared between the two Tibetan paper sample  
326 (Fig.4a, 4b) and the two other samples of *Edgeworthia sp. mitsumata* (Fig.4c) and *Wikstroemia*  
327 *sp. gampi* (Fig.4d). Similitudes in the fingerprints of respective ROI were illustrated by the  
328 presence of stigmastane structures.

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

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



341  
342 **Figure 4.** Total ion current chromatograms of the regions of interest ROI obtained during Py-GC/MS analysis of (a) a  
343 reference modern Tibetan paper sample, (b) an ancient Tibetan paper sample from Samye Monastery, (c) a reference  
344 mitsumata paper sample, (d) a reference gampi paper sample.

### 345 3.4 Py-GCxGC/MS analysis

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

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

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

372 Noticeably, markers of casein which have been previously characterized in proteinaceous binder  
373 in art material were detected during our Py-GC×GC/MS analysis [38, 39]. It was also observed  
374 that animal glue based on collagen addition presented a higher ratio of hydroxyproline during Py-  
375 GC/MS analysis since this latter amino-acid is more represented in collagen than in other  
376 materials like eggs or milk [38, 39]. Conversely, milk does not express much Hyp based DKPs  
377 as noticed here in the Py-GC×GC/MS experiments. To corroborate the Py-GC×GC/MS results,  
378 extracted ion chromatograms for the m/z 154, 210 permitted to check the detection of traces of  
379 DKPs during the Py-GC/MS experiments, confirming so forth the assumption of protein addition  
380 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.

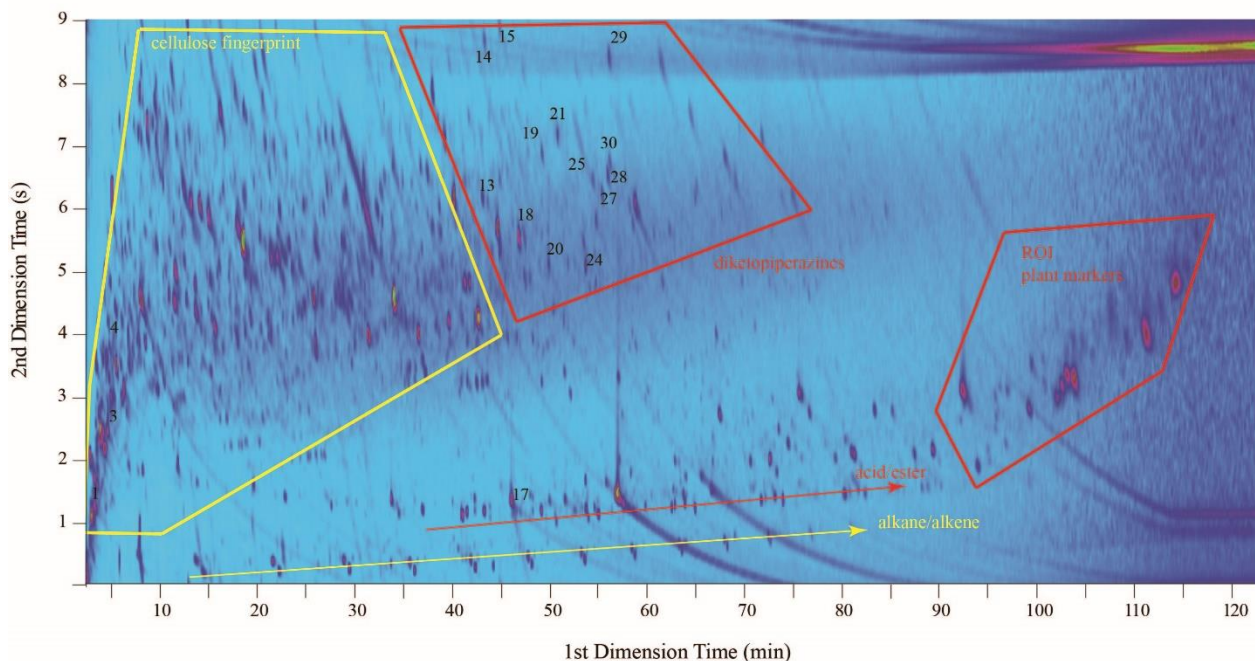
384

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	

385

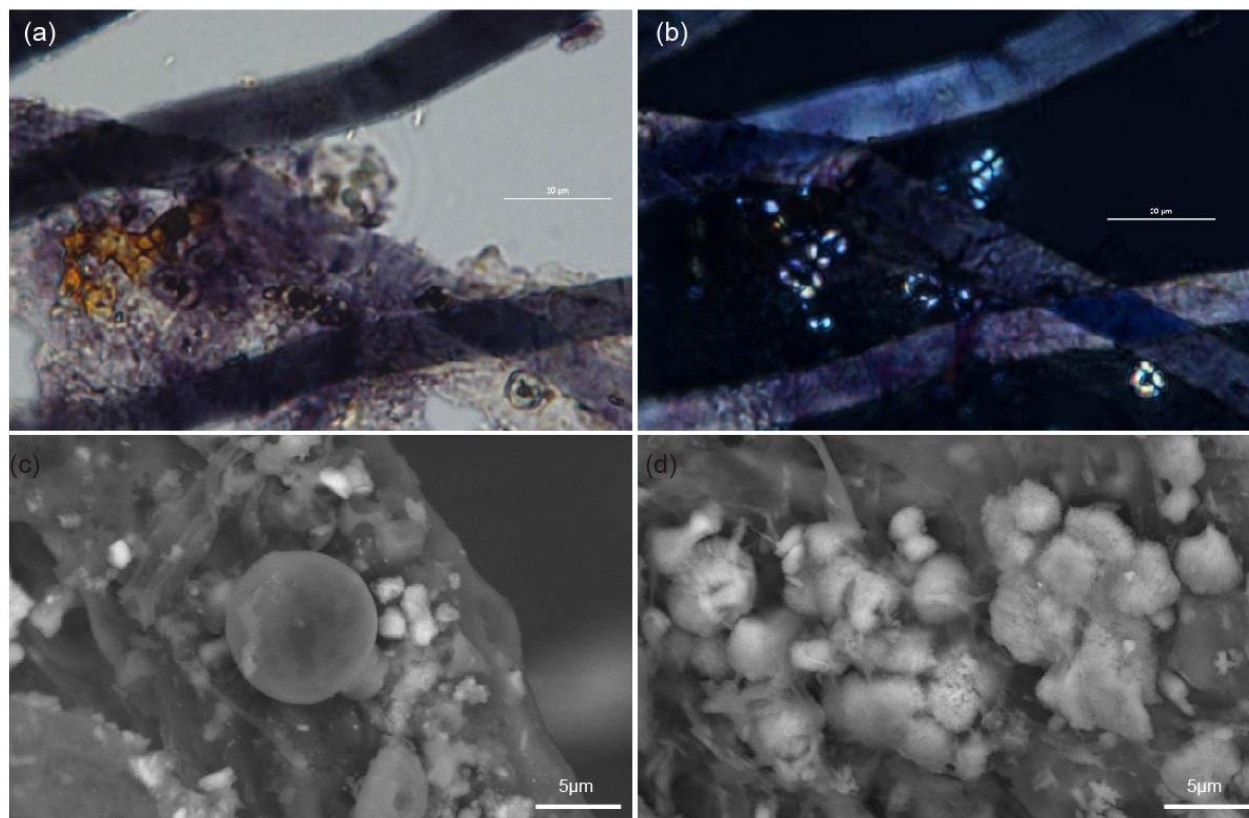




386  
 387 **Figure 5. Py-GC×GC/MS chromatogram of the total ion current of the ancient Tibetan paper sample with labelled casein**  
 388 **peaks reported in Table 2.**

389 **3.5 the filler and sizing agent**

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



407  
 408 **Figure.6 the observation of unaltered starch grains under transmitted light (a) and under polarized light (b) as well as by**  
 409 **SEM (c). (d) is the observation of calcium carbonate particles by SEM. More SEM observation images as well as the EDS**  
 410 **analysis results are provided in Fig.S5, Fig.S6 and Table S1 in Supplementary Information.**

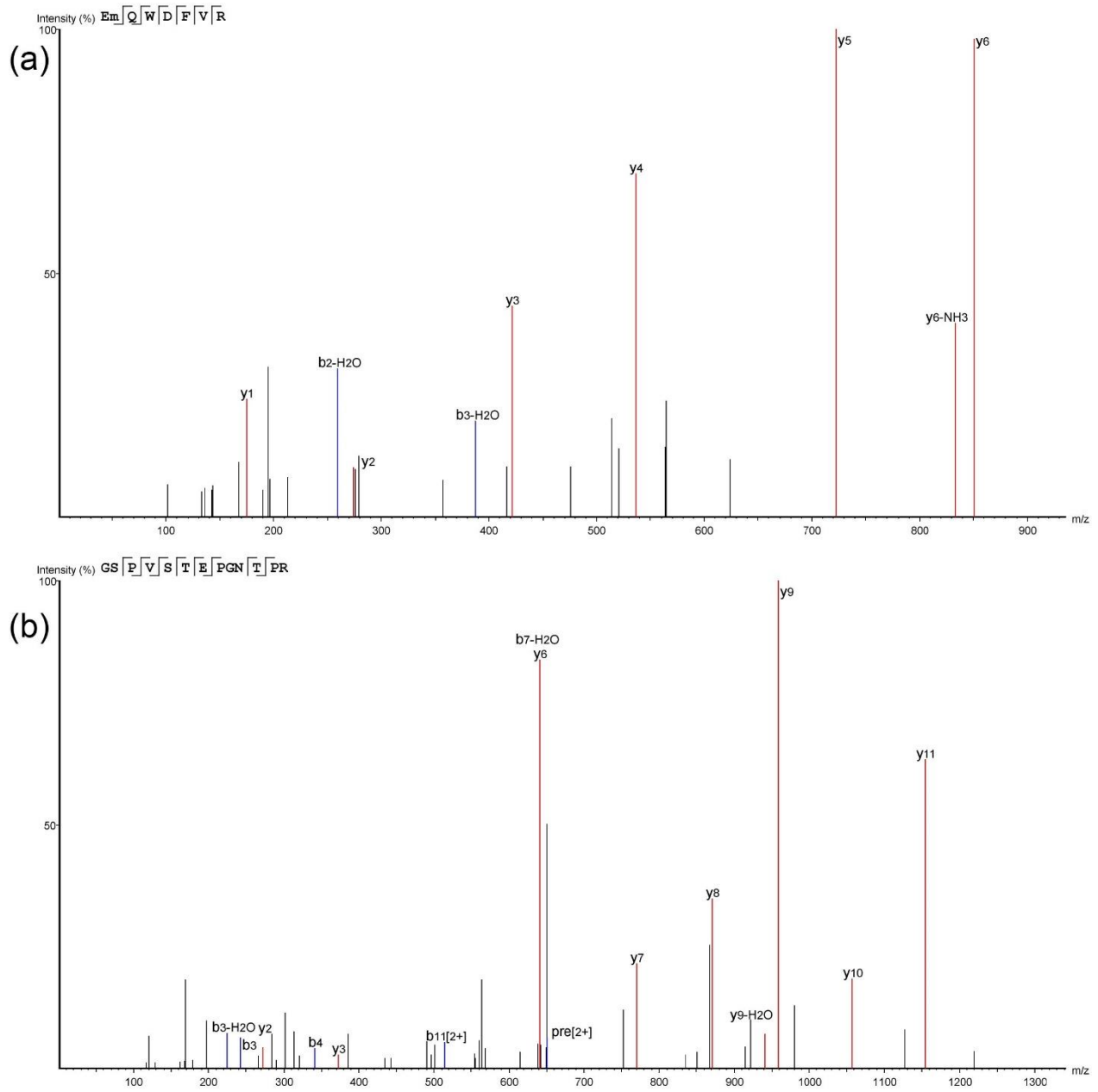
411 The detection of starch grains and paste revealed that starch was mainly used as a sizing agent  
 412 (underwent gelatinization process), exceeding the role of some kind of filler when forming  
 413 agglomerations (the unaltered granules). A sizing agent recipe combining animal glue (not  
 414 detected in Py-GCxGC/MS and Proteomic analysis) and alum ( $KAl(SO_4)_2 \cdot 12H_2O$ ) as precipitant,  
 415 observed in contemporary central China, was less likely used in the treatment of this ancient  
 416 Tibetan fragment. As demonstrated by the EDS results in which aluminum (Al) and sulfur (S)  
 417 were none detected or in trace level (except point 28 and 36 in Fig.S6 and Table S1,  
 418 Supplementary Information, while the coexistence of silicium (Si) may indicate environmental  
 419 sources of these elements).

420 Besides the unaltered starch grain, the possible presence of inorganic fillers was examined by  
 421 conducting energy-dispersive X-ray spectroscopy (EDS) elemental analysis. Large content and  
 422 aggregation of particles has been observed on the surface as well as inside the paper fragment  
 423 under SEM observation ((f)-(i) in Fig.S5, Supplementary Information) and the elemental analysis  
 424 revealed these particles were mainly composed of carbon (C), oxygen (O) and calcium (Ca)  
 425 (Fig.S6, Supplementary Information) which correspond to calcium carbonate ( $CaCO_3$ ), a  
 426 common filler for paper treatment. Thus, the feature for the fillers applied to the paper fragment

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

### 429 **3.6 Proteomic analysis**

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



452  
 453 **Fig.7 MS/MS spectra of two specific peptides. (a) EmQWDFVR from *Triticum spp.*; (b) GSPVSTEPGNTPR from common**  
 454 **wheat (*Triticum aestivum*).**

455 **4. Discussions**

456 **4.1 the use of *Stellera chamaejasme* fiberfor Tibetan papers**

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

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

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

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

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

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

504 It has been noted that the Tibetan *Stellera chamaejasme*, as unique raw material for papermaking,  
505 has not been observed in current examination of Endere and Dunhuang fragments [1].  
506 *Thymelaeaceae* family plant fibres (specifically *Daphne* or *Edgeworthia* sp.) are reported as  
507 scarcely identified in ancient Tibetan manuscripts [53]. The early description of *Stellera*  
508 *chamaejasme* in the Tibetan papermaking could be traced back to 1918 by British consular  
509 officer Eric Teichman: "I came across some Tibetans digging up the root of a kind of bush from  
510 which Tibetan paper is made. This paper is used all over Tibet". *Stellera* was regarded as high-  
511 quality paper and has been used for special purpose. Missionary Duncan also recorded the use of  
512 *Stellera chamaejasme* root for paper making in Dege County during his stay between 1921-1932  
513 [36]. However, Tibetan papermakers likely have preferred to use  
514 *Daphne/Wikstroemia/Edgeworthia* than *Stellera chamaejasme* when possible since this latter  
515 plant is known to necessitate a longer time for processing, in particular, its roots are difficult to be  
516 extracted from the soil, and the whole papermaking process requires an extra step in processing  
517 [36]. Consequently, it explains why *Stellera chamaejasme* is reported to be used in a few printing  
518 houses and papermaking centers.

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

#### 526 **4.2 a new perspective on the way starch sizing agent prepared**

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

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

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

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

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

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

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

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

## 611 **5. Conclusion**



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

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

639

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