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Chemical preservation of tail feathers from *Anchiornis huxleyi*, a theropod dinosaur from the Tiaojishan Formation (Upper Jurassic, China)

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28 **Abstract:** A panel of geochemical techniques is used here to investigate the taphonomy of fossil
29 feathers preserved in association with the skeleton of the Jurassic theropod *Anchiornis huxleyi*. Extant
30 feathers were analysed in parallel to test whether the soft tissues morphologically preserved in the fossil
31 also exhibit a high degree of chemical preservation. Scanning electron microscopy (SEM) and energy
32 dispersive spectroscopy (EDS) indicate that clays and iron oxide pseudomorphs occur in the surrounding
33 sediment and also reveal the preservation of melanosome-like microbodies in the fossil. Carbon gradient
34 along a depth profile and co-occurrence of carbon and sulphur are shown in the fossil by elastic
35 backscattering (EBS) and particle-induced X-ray emission (PIXE), which are promising techniques for
36 the elemental analysis of fossil soft tissues. The molecular composition of modern and fossil soft tissues
37 was assessed from micro-attenuated total reflectance fourier transform infrared spectroscopy (micro-
38 ATR FTIR), solid-state ^{13}C nuclear magnetic resonance (CP-MAS ^{13}C NMR) and pyrolysis gas
39 chromatography mass spectrometry in the presence of TMAH (TMAH-Py-GC-MS). Results indicate
40 that the proteinaceous material that comprises the modern feathers is not present in the fossil feathers.
41 The fossil feathers and the embedding sediment exhibit a highly aliphatic character. However,
42 substantial differences exist between these samples, revealing that the organic matter of the fossil
43 feathers is, at least partially, derived from original constituents of the feathers. Our results suggest that,
44 despite the morphological preservation of *Anchiornis* feathers, original proteins, i.e. keratin, were
45 probably not preserved in the 160-myr-old feathers.

46 **Key words:** *Anchiornis*, fossil feathers, taphonomy, soft tissue preservation, dinosaur.

47

48

49 INTRODUCTION

50 Preservation of soft parts (non-mineralized tissues) in fossil animals is relatively rare when considering
51 the whole geological record. Soft parts of organisms are usually lost during the diverse degradation
52 processes occurring during fossilization. Their constitutive labile organic compounds are usually too
53 fragile to be preserved, compared to the ‘hard’ (biomineralized) parts, which are generally better
54 preserved. However, some important fossil-bearing sites yield not only exquisitely preserved skeletons
55 but also remains of soft tissues, such as skin, scales, hair or feathers (e.g. Allison & Briggs 1993; Zhu *et*
56 *al.* 2005; Pan *et al.* 2013). Feathers, the epidermal appendages that form the external covering of modern
57 birds, have been discovered preserved in close association with fossils of theropod dinosaurs in
58 *Konservat-Lagerstätten* (localities that are characterized by the unusual quality of the fossils) from the
59 Upper Jurassic and Lower Cretaceous of China (Xu *et al.* 1999, 2009, 2012; Hu *et al.* 2009; Godefroit *et*
60 *al.* 2013; Chu *et al.* 2016) and Germany (Rauhut *et al.* 2012). During the last twenty years, Liaoning
61 Province, in north-eastern China, has yielded well-preserved vertebrate fossils with soft parts (e.g.
62 Benton *et al.* 2008; Kellner *et al.* 2010; Li *et al.* 2012). The most striking discoveries were exquisitely
63 well-preserved feathered theropod dinosaurs, evidencing their relationship with modern birds. Since the
64 discovery of the Early Cretaceous *Sinosauropteryx prima* (Ji & Ji 1996), many other feathered
65 specimens have been found (Hu *et al.* 2009; Li *et al.* 2012; Xu *et al.* 2012, 2015). In the same way, the
66 discovery of one of the most primitive birds, *Archaeopteryx lithographica*, associated with well-
67 preserved feathers, constitutes a gigantic step in the comprehension of bird and feather evolution
68 (Christiansen & Bonde 2004). Interestingly, elongate filaments interpreted as primitive feathers were
69 observed in ornithischian, i.e. non-theropod, dinosaurs (Mayr *et al.* 2002; Zheng *et al.* 2009). Recently,
70 both ‘feather-like’ structures and scales were discovered together with remains of the middle Jurassic

71 neornithischian *Kulindadromeus zabaikalicus* collected from volcanoclastic deposits in Siberia
72 (Godefroit *et al.* 2014, 2020; Cincotta *et al.* 2019). Recently, a small theropod dinosaur, the
73 scansoriopterygid *Ambopteryx longibrachium* (Wang *et al.* 2019) from the Upper Jurassic of China, was
74 described with membranous wings instead of feathered ones. This wing configuration was probably lost
75 during evolution in favour of the feathered wing configuration that occurs in modern birds.

76 In a recent study, Zhao *et al.* (2020) observed the structure of experimentally matured feathers and
77 reported the fusion of barbules in the matured feathers. This result has implications in terms of feather
78 taphonomy and evolution, for the absence of barbules in fossil feathers could be, according to the
79 authors, due to their fusion during diagenesis rather than their true absence in the specimen. The addition
80 of chemical analyses to this study would have probably allowed a better understanding of how
81 maturation affects the preservation of biomolecules in feathers.

82 Fossil feathers show a wide range of preservation degrees (e.g. Schweitzer 2011; Xing *et al.* 2016). The
83 study of these diversely preserved structures is crucial for a better understanding of the taphonomic
84 processes leading to their preservation. In most cases, feathers and other types of preserved soft-tissues
85 were deposited in calm, low-energy environments (Kellner & de Almeida Campos 2002). They are
86 found in diverse environmental settings such as shallow-marine (Barthel 1964; Heimhofer & Martill
87 2007; Martill & Heimhofer 2007), lacustrine (e.g. Harms 2002; Zhou *et al.* 2010; Sullivan *et al.*
88 2014), or terrestrial (Manning *et al.* 2013). Different modes of preservation occur for ancient soft
89 tissues: carbonaceous films (e.g. Li *et al.* 2010; Lindgren *et al.* 2015), phosphate (Allison & Briggs
90 1993; Briggs *et al.* 1993), pyrite (Briggs *et al.* 1991; Leng & Yang 2003; Farrell *et al.* 2013), clay

91 minerals (Gabbott *et al.* 2001; Martin *et al.* 2004), aluminosilicates (Butterfield *et al.* 2007) or a
92 combination of these (Wilby *et al.* 1996).

93 Feathers are epidermal appendages mainly composed of keratin (Lucas & Stettenheim 1972), which is
94 present as two secondary structures, alpha-helices and beta-sheets, corresponding to alpha- and beta-
95 keratin, respectively (Fraser & MacRae 2012). Alpha-keratin plays a hydrophobic role in avoiding water
96 loss, whereas beta-keratin increases skin hardness (Gregg & Rogers 1986; Fraser & Parry 1996).

97 According to Lucas & Stettenheim (1972), the amino acid content of keratin in modern bird feathers is
98 rather homogenous in identical parts of a feather (e.g. in rachis of feathers belonging to the same
99 species), although it varies from one species to another. Nonetheless, feather keratin always comprises
100 high amounts of serine, glycine, proline, and lower quantities of valine, leucine, alanine and cysteine
101 (O'Donnell & Inglis 1974; Arai *et al.* 1983, 1986; Gregg & Rogers 1986; Murphy *et al.* 1990; Staroń *et*
102 *al.* 2011; Saravanan & Dhurai 2012).

103 The potential of keratin to resist diagenetic processes is still poorly known. Saitta *et al.* (2017)
104 performed decay and maturation experiments of various keratinous structures, which suggested that
105 feather keratin would not survive diagenesis. Although the ultrastructure of feather keratin, i.e. fibrils,
106 can be preserved (Lindgren *et al.* 2015), there is no direct evidence for the preservation of its
107 proteinaceous compounds. Several immunohistological studies have suggested that keratin could be
108 preserved (Schweitzer *et al.* 1999; Moyer *et al.* 2016; Pan *et al.* 2016), although this method remains
109 highly controversial. By contrast, melanin (the natural pigment present in a variety of soft tissues
110 including hair, skin and feathers) is considered to be more resistant to degradation and fossilization
111 processes. Melanin has been unequivocally identified in various types of fossil tissues, such as fish eyes

112 (Lindgren *et al.* 2012), bird feathers (Colleary *et al.* 2015), non-avian dinosaur feathers (Lindgren *et al.*
113 2015), mammal hair (Colleary *et al.* 2015) and frog skin (McNamara *et al.* 2016).

114 Here, we investigate the ultrastructure and chemical composition of fossil feathers of a theropod
115 dinosaur, *Anchiornis huxleyi* (YFGP-T5199), collected from Upper Jurassic deposits of the Tiaojishan
116 Formation (Liaoning Province, China). Previous study of the same specimen focused on the
117 identification of pigment remains (eumelanin), and evidenced the preservation of melanosomes in the
118 feathers (Lindgren *et al.* 2015). We report new and complementary geochemical information about the
119 preservation of macromolecular compounds in the fossil feathers using a range of analytical tools. The
120 surrounding sediment and modern feathers were analysed in parallel to ascribe pristine constituents.

121 Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS) were used to
122 identify and characterize the elemental composition of preserved pigment organelles and the
123 sedimentary matrix. X-ray diffraction (XRD) was used to analyse the mineralogical composition of the
124 samples in an attempt to understand the role of sediment mineralogy in the preservation of soft tissues.

125 Ion beam analysis (IBA), is recognized as a promising archaeometric tool (Jeynes & Colaux 2016) and
126 has recently been successfully applied to human bone analyses (Beck 2014). In this work, particle-
127 induced X-ray emission (PIXE) and elastic backscattering spectrometry (EBS) were used for the first
128 time on fossil soft tissues to get insights into the heavy (PIXE) and light (EBS) in-depth elemental
129 composition of the samples. This approach is innovative in the study of organic materials. Organic
130 geochemistry techniques, micro-attenuated total reflectance fourier transform infrared spectroscopy
131 (micro-ATR FTIR), ¹³C-Nuclear Magnetic Resonance (NMR), Pyrolysis gas chromatography-mass
132 spectrometry in the presence of TMAH (TMAH-Py-GC-MS), were applied to characterize the functional

groups and other biomolecular components present in the studied samples. To our knowledge, the detailed chemical characterization by ^{13}C NMR and Py-GC-MS of fossil feathers from a non-avian dinosaur has not been done elsewhere.

MATERIAL AND METHODS

Specimen information

The studied specimen, *Anchiornis huxleyi* (YFGP- T5199) (Fig.1), is a basal Avialan (the description of the specimen is available in Lindgren *et al.* 2015, SI, pp. 18–23) that was collected from the Tiaojishan Formation in the Yaolugou locality (Liaoning Province, China), and belongs to the Yizhou Fossil and Geology Park in Liaoning. The Tiaojishan Formation consists of hundreds of meters of alternating sedimentary and volcanic beds (Yuan *et al.* 2005; Yang *et al.* 2006; Liu *et al.* 2012). Absolute dating on a laterally equivalent formation, the Lanqi Formation, indicates an age ranging between 165.0 ± 1.2 and 153.0 ± 2.0 Ma (Zhang *et al.* 2008; Chang *et al.* 2009), which spans the Callovian-Kimmeridgian interval (Middle–Late Jurassic; Gradstein *et al.* 2012). YFGP- T5199 is embedded in thinly laminated carbonate sediments, corresponding to alternation of very thin marl and thicker clay laminae. These sediments were deposited in the context of a lake affected by episodic volcanic eruptions (Nan *et al.* 2012). Recent U–Pb radiochronological analyses on zircons from the Jianchang locality indicate that the Yanliao Biota, that includes *Anchiornis* as well as pterosaurs and eutherian mammals, is Oxfordian in age (Chu *et al.* 2016). The plumage of the specimen studied herein is morphologically preserved as dark brown residues around the skeleton, especially around the tail and the forelimbs, and on the skull.

154 *Sample description*

155 The studied samples consist of fossil feather fragments dissected from the posterior end of the tail (Fig.
156 1, the dark area in the white box, top right) of YFGP- T5199, as well as fragments of the host sediment
157 (Fig. 1, the light area in the white box). To test for possible chemical contamination of the fossil feathers
158 by sediment, the sediment samples were analysed using the same methodology as for the fossil feathers.
159 Two types of sediment samples were studied: (1) ‘host’ sediment directly in contact with the feathers
160 from the tail (light area in white box on Fig. 1); and (2) ‘remote’ sediment located > 100 mm from the
161 fossil on the same slab (yellow box bottom left on Fig. 1).

162 *Sample preparation*

163 Two modern brown wing feathers of *Buteo buteo* (buzzard, Aves: Accipitriformes; RBINS collection
164 number: A4011A01; Cincotta *et al.* 2020, fig. S1) were analysed for comparative purposes. Different
165 parts of the feathers, rachis and barbs, were analysed with IBA. The modern feathers come from a
166 specimen that died naturally and was stored at –18°C at the Royal Belgian Institute of Natural Sciences
167 prior to analysis.

168 Two types of sample were collected on the fossil specimen: millimetre-sized samples for SEM and EDS,
169 as well as centimetre-sized samples for the other analytical approaches. We took a total of 12 millimetre-
170 sized samples (Fig. 1) from different regions of the body of YFGP-T5199 with a sterile scalpel. The
171 samples were mounted on double-sided carbon tape and sputter-coated with gold (BAL-TEC SCD 050).
172 Centimetre-sized fragments of approximately 5 mm² and 2 mm thick, from fossil feathers (white box in
173 Fig. 1) and sediment (yellow box in Fig. 1) were dissected with a sterile scalpel. Samples were cleaned
174 with distilled water without any additional preparation prior to analysis.

175 Several points were analysed with IBA and micro-ATR FTIR on two centimetre-sized fragments
176 containing both fossil feathers and their ‘host’ sediment, and one fragment of ‘remote’ sediment
177 (Cincotta *et al.* 2020, figs. S1B, S2). Other centimetric samples from the same region were collected for
178 ^{13}C NMR and Py-GC-MS (white and yellow boxes on Fig. 1). These samples were crushed and lipids
179 were extracted in order to: (1) eliminate potential contaminants related to sample manipulation; and (2)
180 concentrate macromolecular organic matter which mainly corresponds to proteins in modern feathers.
181 Samples were ground to a fine homogeneous powder in an agate mortar. Lipid extraction involved three
182 successive ultrasonifications (10 min) in 15 ml of dichloromethane/methanol (2:1, v/v), at room
183 temperature and centrifugation at 1921 g (10 min). The supernatant was removed and the pellet was
184 dried under nitrogen and stored in the dark at 5°C prior to analysis.

185 *Analytical methods*

186 Samples were imaged under low vacuum with an environmental QUANTA 200 (FEI) scanning electron
187 microscope (at an acceleration voltage ranging from 20 to 30 kV and working distances of 8 – 15 mm).
188 Subsequent semi-quantitative EDS analyses (single point and mapping) were performed using either an
189 environmental QUANTA 200 (acceleration voltage of 30 kV and working distance of 10 mm) or a field-
190 emission JEOL 7500F (acceleration voltage of 15 kV and a working distance of 8 mm).

191 XRD analyses were carried out on both bulk rock and clay mineral with a Philips diffractometer using
192 Cu K_α radiation. A tube voltage of 40 kV and a tube current of 30 mA were used. The goniometer
193 scanned from 3° to 70° (2 θ) for the bulk rock and from 3° to 30° (2 θ) for clay minerals. The clay
194 minerals (<2 μm fraction) were isolated by successive centrifuging after decarbonation of the crushed
195 rock with 1N HCl. The preparation was mounted on glass slides and treated according to the three

196 following protocols: (1) natural (air-dried); (2) ethylene-glycol solvation; and (3) heated at 490°C for 2
197 h. Clay minerals were identified according to the position of the (001), or (0001), series of basal
198 reflections on the X-ray diffractograms.

199 EBS and PIXE measurements were performed using a 3 MeV proton (^1H) beam from the Tandetron
200 linear accelerator ALTAÏS (University of Namur). PIXE is highly sensitive to Na to U elements whereas
201 EBS signals are enhanced for light elements (e.g. C, N and O) due to strong non-Rutherford cross-
202 sections. These two integrative methods, together, can identify almost all elements of the periodic table.
203 The beam spot size was reduced to 0.5 mm in diameter to minimize topographic effects. Backscattered
204 particles were detected using two detectors mounted at scattering angles of 170° and 165°, whereas the
205 emitted X-rays were collected with an Ultra-LEGe (ultra low energy germanium) detector mounted at
206 135°. Angles are given relative to the incident beam direction. A selective filter (6 µm of Al) was
207 mounted in front of the Ultra-LEGe detector to lower the strong Si signal and therefore enhance the
208 rather weak S signal observed in the fossil feathers. The modern brown feathers were analysed at two
209 different locations (barb and rachis; Cincotta *et al.* 2020, figs. S1, S2). Two locations were analysed in
210 the fossil feathers. The ‘host’ sediment was analysed at three different locations (at 1.7, 3.2, and 4.8 mm
211 away from the fossil) and the ‘remote’ sediment at one location (Cincotta *et al.* 2020, fig. S2). All the
212 samples were analysed using the same experimental settings. A certified reference material (BCR-126A
213 lead glass from NIST) was analysed to: (1) calibrate the detectors (both EBS and PIXE); and (2)
214 estimate the accuracy of the PIXE measurements. The EBS spectra were analysed with DataFurnace
215 software (Jeynes *et al.* 2003) together with the cross-sections generated by SigmaCalc (Gurbich 2016) to
216 derive the depth profiles of the major elements (for C, see Cincotta *et al.* 2020, fig. S4). The integral of
217 C, O and Si depth profiles (integration limits set to 0–25 000 TFU¹, or 0–3 µm considering a density of

218 2.65 g/cm³ for the sediment) yields the C, O and Si equivalent thicknesses given in TFU (details on the
219 global uncertainty calculations can be found in Cincotta *et al.* 2020, table S1). The PIXE spectra were
220 manipulated with GUPIX software (Campbell *et al.* 2010). The matrix composition to be used in GUPIX
221 was determined by integrating the depth profiles of the main components observed by EBS (i.e. C, O
222 and Si) on a given interval (0–100 000 TFU).

223 Micro-ATR FTIR was performed on modern and fossil feathers using a Bruker Vertex 70 FTIR
224 spectrometer (University of Hasselt, Belgium) equipped with a Hyperion 2000 microscope and MCT
225 detector. The infrared spectra were collected in the mid-IR range, from 4000 to 600 cm⁻¹, and 32 scans
226 were acquired in attenuated total reflectance (ATR) mode (Ge –ATR crystal) with a resolution of 4 cm⁻¹.
227 FTIR spectroscopy was used on a modern buzzard feather (dark regions), one sample of fossil feather
228 from the tail of *A. huxleyi* and the surrounding sediment, to identify the presence or absence of
229 functional groups in their molecular composition.

230 ¹³C NMR is a spectroscopic method that documents the chemical environment of carbon in organic
231 compounds. Solid state ¹³C NMR spectra of the lipid-free samples were obtained at 125 MHz (Bruker
232 Avance 500 spectrometer) using a 4 mm zirconium rotor, with a cross-polarization (CP) sequence and
233 magic angle spinning (MAS) at 14 kHz. CP-MAS ¹³C NMR spectra were acquired with contact time of
234 1 ms and recycle time of 1s (fossil and sediment) or 3s (modern feathers). The use of a single contact
235 time does not allow precise quantification of the identified chemical functional groups. Each spectrum
236 was the result of 6 000 (modern samples) to 400 000 (sediments) scans.

237 Curie point Py-GC-MS gives insight into the molecular composition of organic macromolecular
238 materials through their thermal degradation into molecular building blocks that can be separated by gas

239 chromatography (GC) and further identified by mass spectrometry (MS). Tetramethylammonium
240 hydroxide (TMAH) was used to enhance the thermal breakdown of macromolecules and induce *in situ*
241 methylation of pyrolysis products, which, in turn, enhance their detection and identification in GC-MS.
242 The samples were mixed with an excess of TMAH (25 wt % in methanol) in a 1:1 (wt/wt) ratio before
243 loading in ferromagnetic tubes with Curie temperature of 650°C. Masses of 2 mg were used for the
244 modern samples, 6 mg for the fossil feathers, and 16 mg for the sediment. Curie point pyrolysis was
245 carried out with a Pilodist Curie flash pyrolyser. Samples were heated at their Curie temperature for 10 s
246 under a He flow of 1 ml/min. The instrument was coupled directly to a GC-MS system. The pyrolysis
247 products were separated using a Trace Thermo gas chromatograph equipped with a Rxi5SilMS column
248 (30 m × 0.25 mm i.d., 0.5 µm film thickness). Helium was used as carrier gas at constant pressure of 15
249 psi. The injector temperature was 280°C in spitless mode. The oven temperature was maintained at 50°C
250 for 10 min and was progressively increased to 310°C at 2°C/min. Coupled to the gas chromatograph was
251 a DSQ Thermo mass spectrometer with a heated interface (310°C), electron energy of 70 eV and ion
252 source at 220°C, scanning from m/z 35 to 800 at 2 scans/s. Compounds were assigned on the basis of
253 their mass spectra, comparison with the NIST library mass spectra, published mass spectra (Gallois *et al.*
254 2007; Templier *et al.* 2013) and GC retention times. The molecular structure of all compounds present in
255 substantial amount was investigated without any ion selection that could have biased interpretations.

256 *Institutional abbreviation.* NIST, National Institute of Standards & Technology, US Department of
257 Commerce; RBINS, Royal Belgian Institute of Natural Sciences, Brussels, Belgium; YFGP, Yizhou
258 Fossil & Geology Park, China.

259

RESULTS

SEM/EDS and XRD

SEM of the fossil feathers revealed that they are embedded in a sedimentary matrix containing mainly quartz, carbonates, and phyllosilicates. The latter are organized in thin platelets oriented parallel to each other (Fig. 2A, B). A feather sample from the right wing of *Anchiornis* (sample 1 on Fig. 1) showed abundant rounded crystals that are present only beneath the surface. They occur mainly as framboids (Fig. 2C), but also as individual microcrystallites (Fig. 2D) and, in some cases, are associated with voids. Framboids are spheroidal or ovoid, 6–9 μm in diameter and contain dozens of euhedral crystals, 750 nm in diameter. In contrast, individual cubic crystals are much smaller (about 500 nm³) and contain micro-crystallites (Fig. 2D). EDS analyses indicate that the sediment is composed of Fe, Si, O, Al, C, Ca (and Mn, K, Mg), probably indicating the presence of quartz, calcite, and various phyllosilicates. XRD analyses confirmed the presence of these minerals in the sediment (Fig. 3A). In addition, the XRD spectrum of the < 2 μm phase shows that expansive material, such as interstratified illite/smectite, is present in the sediment (Fig. 3B). Due to their characteristic framboidal shape and elemental composition, the crystal clusters observed beneath the fossil feather surface are attributed to diagenetic iron oxides or hydroxides. Indeed, although the framboidal habit is common for iron sulphides, the lack of S here shows they are rather iron oxide pseudomorphs probably resulting from the *in situ* weathering of pyrite framboids (Nordstrom 1982; Kaye *et al.* 2008; Wang *et al.* 2012; Blanco *et al.* 2013). These structures are associated with thin clay overgrowths (arrow in Fig. 2C, E), indicating that the iron oxides (or the preceding pyrites) precipitated first. Tiny stellate minerals were also observed and identified as

281 probable iron oxides by X-ray spectroscopy (Fig. 2F). The presence of calcium carbonates, feldspars
282 (Fig. 2G) and quartz (Fig. 2H) in the sedimentary matrix was confirmed by X-ray spectroscopy.

283 Elongate microbodies, 650–950 μm , and their associated imprints were observed in three samples (6, 9,
284 and 12 on Fig. 1) collected from the anterior and posterior parts of the dinosaur tail (Fig. 4). Microbody
285 imprints are abundant, tightly packed together and randomly oriented (Fig. 4A, B). They probably
286 represent traces of melanosomes. Similar microbodies interpreted as melanosomes have previously been
287 observed in feathers from the crest of this specimen (YFGP-T5199; Lindgren *et al.* 2015). Here, isolated
288 elongate structures were observed (Fig. 4C). These fossil organelles are preserved within the thin clay-
289 rich sediment.

290 *Ion Beam Analysis: EBS and PIXE*

291 The great virtue of EBS is to be capable of yielding the elemental depth profiles non-destructively from
292 the outermost microns of the sample with good sensitivity and depth resolution (Jeynes & Colaux 2016).
293 A typical EBS spectrum obtained from the fossil feathers is shown in Cincotta *et al.* (2020, fig. S3)
294 together with its best fit. The experimental spectrum was inverted to recover the elemental depth profiles
295 (examples are shown for carbon in Cincotta *et al.* 2020, fig. S4). Integration of these elemental depth
296 profiles allows derivation of the concentration of each element at a given depth. Figure 5A clearly shows
297 that the C enrichment in the near surface region (*c.* 60%) decreases at increasing distance from the fossil
298 feathers, reaching a minimum in the ‘remote’ sediment (*c.* 14%). Sample concentrations of O and Si
299 (although less obvious) follow an opposite trend. The N content in the modern buzzard feathers is *c.* 20–
300 26%. The very low content of N in the fossil (*c.* 5%) and even less (under the limit of detection) in the
301 remote sediment (Cincotta *et al.* 2020, fig. S3) precluded its depth profiling.

302 In contrast, EBS analysis of modern buzzard feathers shows homogeneous concentrations with depth
303 (Cincotta *et al.* 2020, fig. S5). In the buzzard feathers, C content is about 60 at.%, while N and O are
304 both around 20 at.% for the rachis and around 25 and 15 at.% respectively for the barbs. Typical PIXE
305 spectra acquired from the fossil feathers, ‘host’ sediment and ‘remote’ sediment are shown in Figure 5B.
306 The samples differ in the amounts of several elements present (Cincotta *et al.* 2020, tables S1, S2). Of
307 particular interest is the S content: there are elevated concentrations of S in the fossil feathers (average
308 1842 ± 208.5 ppm), less in the ‘host’ sediment (1162 ± 143 ppm), and much lower concentrations in the
309 ‘remote’ sediment (98 ± 35 ppm). The concentration of S in the fossil feathers is roughly 20 times lower
310 (*c.* 0.2 wt.%) than in the modern bird feathers (*c.* 3.7–4.3 wt.%). The co-occurrence between S and C is
311 also highlighted (Table 1). Both concentrations decrease with depth within the fossil feathers whereas
312 the reverse situation is observed for Si and O.

313 ¹³C NMR

314 The ¹³C NMR spectrum of buzzard feathers (Fig. 6A) shows a complex signal in the aliphatic region,
315 with well-resolved peaks between 10 and 65 ppm and a narrow peak at 173 ppm, due to carboxyl
316 carbons, i.e. carboxylic groups and esters, and amides. Two additional, less intense, signals can be seen
317 at 129 ppm and 158 ppm in the unsaturated/aromatic carbon region. In comparison to the spectrum of
318 modern feathers, the ¹³C NMR spectra of the fossil feathers and their surrounding sediment show much
319 simpler patterns (Fig. 6B, C). The spectra are similar to each other and both are dominated by a broad
320 peak in the aliphatic region, maximizing at 30 ppm and thus indicative of long alkyl chains. Two
321 additional broad signals contribute to the spectra. The first one occurs as a broad shoulder between 68
322 and 80 ppm, in the O-alkyl C and N-alkyl C range, and the second one is a broad peak at 129 ppm,
323 attributed to aromatic carbons.

324 *Micro-ATR FTIR spectroscopy*

325 The micro-ATR FTIR spectra of the theropod feathers, the embedding sediment and the modern buzzard
326 feather (dark regions) are shown in Figure 7.

327 The spectrum of the dark region of a modern buzzard feather shows characteristic bands of secondary
328 amides (as in proteins and polypeptides) at 1628 cm^{-1} (C=O stretch of Amide I), 1531 cm^{-1} (C–N
329 stretch, Amide II) and 1239 cm^{-1} (N–H in plane bending coupled with C–N stretch, Amide III). Broad
330 bands around 3274 and 3125 cm^{-1} can be attributed to the N–H stretching of secondary amides. The
331 bands at 2961 , 2922 and 2852 cm^{-1} are assigned to the C–H stretching of methylene and methyl groups.
332 These two spectra are very similar and no significant differences could be found between IR response of
333 the dark and white regions of the same feather.

334 The spectrum of the fossil feathers has a different pattern but some similarities with the modern buzzard
335 feather appear. The distinct bands at 2920 and 2851 cm^{-1} can also be attributed to C–H stretching of
336 methylene and methyl groups. These associated bands are also present in the IR spectrum of the
337 surrounding matrix but with different relative intensities. A broad region around 3300 cm^{-1} is present,
338 although much less marked, and is indicative for O–H stretching as found in carboxylic groups and
339 alcohols. In the spectra of the fossil feathers, a broad band at about 1560 cm^{-1} can be attributed to
340 carboxylate. This band is not present in IR spectrum of the sediment. Another broad band around 1412
341 cm^{-1} is found in the spectrum of the fossil feathers and might be related to the presence of CaCO_3
342 overlapping with C–H bending vibrations at 1460 and 1380 cm^{-1} (Andersen & Brečević 1991; Kröner *et*
343 *al.* 2010; Kiros *et al.* 2013) . This is also confirmed by the presence of weak bands at 873 and 718 cm^{-1} .
344 The IR spectrum of the sediment shows a similar, but less defined, absorption band between 1415 and

1463 cm⁻¹. The IR spectrum of the fossil feathers and its surrounding matrix both show a narrow band at 1260 cm⁻¹ that could be attributed to the Si-CH₃ vibrations. Both spectra show an intense broad band at 1013 cm⁻¹ together with the weaker bands at 873 and 797 cm⁻¹ related to the sedimentary matrix (clay minerals, quartz, silicates, Si-O stretching). The IR spectrum of the sedimentary matrix shows an additional band at 720 cm⁻¹ that is also present, although very weak, in the fossil, indicating long-chain alkyl groups (CH₂ rocking vibrations). All the peaks mentioned above are absent in the IR spectra of the modern feather.

TMAH Py-GC-MS

Pyrochromatograms were obtained for the three following samples: the modern and fossil feathers, and the 'host' sediment. In agreement with previous studies on bird feathers (Brebü & Spiridon 2011; Saitta *et al.* 2017), the pyrochromatogram of the modern feathers is dominated by cyclic molecules containing N, along with methylbenzene **1**, methylbutane nitriles **2**, **3** and cyclohexanedione **11** derivatives (Fig. 8A). Detailed interpretation of mass spectral fragmentation patterns allowed identification of the major pyrolysis products (Table 2) and further assignment to possible source. Molecular structures are given in Appendix 1, with methyl groups added by TMAH indicated in bold. Products **6**, **8**, **10** result from direct methylation of alanine, valine and proline, thus pointing to a proteinaceous origin for the feathers. This is further supported by the occurrence in substantial amounts of alkylnitriles **2**, **3** resulting from dehydration of amides involving isoleucine and leucine, respectively, and of methoxybenzenes **7**, **9** released through homolysis of the side chain of tyrosine (Ratcliff *et al.* 1974). Methylbenzene **1**, pyrrole **4** and ethylbenzene **5** are rather ubiquitous pyrolysis products in sedimentary organic matter. However, they can also be released upon pyrolysis of phenylalanine and serine (Gallois *et al.* 2007). Mass spectral

366 fragmentation pattern (base peak at m/z 82) suggests an origin from the side chain of histidine for
367 compound **16**. Similarly, compound **17** probably corresponds to a valine derivative as its mass spectrum
368 is characterized by the loss of 42 amu (i.e. valine side chain). Dimethylcyclohexanedione **11** was
369 reported as pyrolysis product of glycine (Moldoveanu 2009). Glycine is also present as its
370 diketopiperazine **15** resulting from combined dehydration and cyclisation (Simmonds *et al.* 1972). The
371 same mechanism involving two different amino acids (isoleucine-glycine) leads to another
372 diketopiperazine **18** (Hendricker & Voorhees 1996). The formation of more complex diketopiperazines
373 was proposed by Templier *et al.* (2013) from tripeptide units. A similar mechanism can be invoked for
374 the formation of compound **19** from valine, as well as compounds **20** and **21** from serine and leucine
375 (Table 2; Cincotta *et al.* 2020, fig. S6). Imidazolidinedione **12** probably results from the internal
376 cyclization of tripeptide comprising an alanine unit as reported by Templier *et al.* (2013; Cincotta *et al.*
377 2020, fig. S6). The formation of imidazolidinone **13** can be related to the decomposition of bicyclic
378 amidine derived from valine as suggested by Basiuk & Navarro-González (1997; Cincotta *et al.* 2020,
379 fig. S6). Another decomposition pathway of bicyclic amidine is probably responsible for the formation
380 of imidazolidinone **14** from valine and possibly glycine (Templier *et al.* 2013). As far as we know, this
381 is the first identification of such complex molecules (diketopiperazines from tripeptide and
382 imidazolidinone from bicyclic amidine) in the pyrolysate of a natural sample.

383 By comparison, pyrochromatograms of the fossil feathers and their ‘host’ sediment are simpler. They are
384 dominated by *n*-alkane/*n*-alkene doublets (Fig. 8B, C), resulting from the homolytic cleavage of long
385 alkyl chains. In the fossil feathers, these doublets comprise from 8 to 30 carbon atoms, and exhibit a
386 smooth distribution except an intense C₁₈ doublet. An additional series of fatty acid methyl esters with
387 alkyl chain ranging from C₈ to C₃₀ and maximizing at C₁₆ is also identified (Table 2). It results from the

388 release upon pyrolysis of a series of fatty acids that are methylated thanks to TMAH. In addition to these
389 series, a methoxybenzene substituted by two methyl groups or an ethyl group **22** is detected in minor
390 amounts, at the beginning of the pyrochromatogram. A trimethylbenzene and a methylated derivative of
391 methoxyaniline **23** also contribute to this part of the pyrochromatogram. However, the most prominent
392 pyrolysis product **24** corresponds to the C₁₈ alcohol methylated through TMAH pyrolysis.

393 The pyrochromatogram of the “host” sediment shares several similarities with that of the fossil feathers.
394 Indeed, it is dominated by series of alkane/alkene doublets and fatty acid methyl esters. Although the
395 distribution of the fatty acid methyl esters is similar in both samples, that of the doublets differs. Indeed,
396 whereas their range (C₈–C₃₀) is similar, the maximum of the series appears at C₁₅ in the sediment,
397 instead of a marked predominance of the C₁₈ in the fossil (Table 2). Moreover, when comparing the
398 minor compounds eluting at the beginning of the pyrochromatogram, compounds **22** and **23** are common
399 in both samples, whereas a higher number of alkylbenzene homologues occurs in the sediment. Finally,
400 the contribution of octadecanol **24** is much lower in the sediment pyrolysate.

401

402 **DISCUSSION**

403 *Ultrastructure*

404 Microbodies and elongate moulds are observed in feather samples collected at three different locations
405 on *Anchiornis* tail. The elongate shape, parallel orientation and location of the microbodies within the
406 feathers strongly suggest that they correspond to eumelanosomes. These pigment organelles are
407 associated to brown, grey and black hues in modern bird feathers. The preservation of melanosomes, and

408 especially eumelanosomes, in dinosaur feathers is not uncommon. Such microscopic melanin-bearing
409 structures have been described in other theropod dinosaurs, basal birds and isolated feathers (Li *et al.*
410 2010, 2012; Zhang *et al.* 2010; Carney *et al.* 2012; Colleary *et al.* 2015; Pan *et al.* 2016; Hu *et al.* 2018).
411 The chemical composition of these microbodies has been assessed in a previous study, confirming a
412 melanosome origin (Lindgren *et al.* 2015).

413 Recent taphonomic experiments in abiotic conditions suggest that the preservation of mouldic
414 melanosomes requires interaction with an oxidant prior to maturation, and that the preservation of
415 melanosomes is probably less frequent than the preservation of keratinous structures in fossil feathers
416 (Slater *et al.* 2020). Interestingly, *Anchiornis* feathers contain both melanosomes and moulds. The nature
417 of the former experiments does however not reflect the depositional and fossilization conditions of
418 *Anchiornis*.

419
420 *Depth profiling, light and heavy element composition*

421 The C and N concentrations determined by EBS led to N/C ratios of 0.33–0.42 in modern buzzard
422 feathers, and of 0.08 for the fossil, suggesting a marked relative decrease in N. The C concentration
423 gradient observed by EBS in the fossil feathers of *A. huxleyi* strongly suggests that they are preserved as
424 carbonaceous layers located at the uppermost part of the sample (i.e. 0–3 μm depth, given a rock density
425 of 2.65 g/cm³) and suggests that fossil organic matter could have impregnated the sediment only in a
426 nearby area.

427 The PIXE spectra show elevated concentrations of S in the fossil feathers and, to a lesser extent, in the
428 ‘host’ sediment, together with very low concentrations in the ‘remote’ sediment. This suggests that S is

429 associated with the soft tissues. The fossil feathers are therefore preserved as a S-rich carbonaceous film.
430 Substantial quantities of S are present in the modern buzzard feathers ($43\,070 \pm 4236$ ppm in the brown
431 barbs and $37\,142 \pm 3652$ ppm in the rachis, Table 1). This is not surprising due to the presence of S-
432 containing biomacromolecules, such as the pigment phaeomelanin and proteins containing cysteine or
433 methionine (i.e. keratins) in bird feathers (Harrap & Woods 1964; Bortolotti 2010; Murphy *et al.* 1990;
434 Cesarini 1996; Riley 1997; Saravanan & Dhurai 2012). Important studies on the chemical composition
435 of feathers have shown that S is a major element of bird feathers (Harrap & Woods 1964, 1967; King &
436 Murphy 1987; Murphy *et al.* 1990; Edwards *et al.* 2016). Some authors could even discriminate between
437 organic S originating from keratin and phaeomelanin based on its speciation (Edwards *et al.* 2016).
438 Previous *in situ* chemical analysis (time-of-flight secondary ion mass spectrometry; TOF-SIMS) of the
439 melanosomes from the present fossil revealed their enrichment in S with respect to the surrounding
440 sediment, but it could not determine whether it reflects the occurrence of phaeomelanin or diagenetic
441 incorporation of S in eumelanin (Lindgren *et al.* 2015). It has been suggested that divalent elements (Cu,
442 Ca, Zn) formed chelates with melanin in a Cretaceous early bird (Wogelius *et al.* 2011). Such a
443 complexation may have played a role in S preservation in fossil soft tissues. Alternatively, the presence
444 of S in the fossil feathers can be attributed to natural sulphurization of the organic matter, that is the
445 abiogenic intra-molecular incorporation of sulphur from the depositional environment during early
446 diagenesis. The incorporation of S into organic matter was interpreted as a way to enhance the
447 preservation potential of certain labile substances through cross-linking (Sinninghe-Damsté & De
448 Leeuw 1990; Sinninghe-Damsté *et al.* 1989, 1988; McNamara *et al.* 2016). Indeed, organic matter has
449 the ability to form complexes with inorganic elements, including S, which was traced in fossil soft
450 tissues (Wogelius *et al.* 2011).

451 *Functional groups in the organic matter*

452 On the whole, the ^{13}C NMR and IR spectra of buzzard feathers (Figs 6A, 7A) are comparable to that of
453 several keratinous materials, such as feather keratin (Kricheldorf & Müller 1984; Barone *et al.* 2005;
454 Wang & Cao 2012, Sharma *et al.* 2018), wool keratin (Yoshimizu & Ando 1990; Wojciechowska *et al.*
455 2004) or gecko setae keratin (Jain *et al.* 2015). Indeed, the peak at 173 ppm should mainly correspond to
456 the signal of secondary amide ($\text{O}=\text{C}-\text{NH}$) groups involved in the peptidic bonds. This is confirmed by
457 the presence of characteristic bands of secondary amides in the IR spectrum of the brown feathers (Fig.
458 7A). This includes a broad band around 3277 cm^{-1} attributed to the N–H stretching band of amides, a
459 narrow band at 1628 cm^{-1} related to the C=O stretch of Amide I, a band at 1518 cm^{-1} attributed to
460 Amide II and a band at 1237 cm^{-1} related to Amide III (Bendit 1966; Yu *et al.* 2004; Wang & Cao 2012;
461 Giraldo *et al.* 2013; Tesfaye *et al.* 2017).

462 Carbon atoms bearing both COOH and NH_2 groups (termed C_α) in the amino acids (except glycine)
463 resonate between 50 and 60 ppm in ^{13}C NMR. They account for the peaks at 52.9 and 60.2 ppm in the
464 broad aliphatic signal, whereas the signal at 42.6 ppm is assigned to the C_α of glycine. The other peaks
465 are mainly associated with the amino acid side chains, that at 30.8 ppm being assigned to C_β along with
466 C in long alkyl chains, and those at 19.8 and 25.7 ppm to C_γ and C_δ . The three bands located in the
467 $2961\text{--}2850\text{ cm}^{-1}$ range in the IR spectra confirms the presence of aliphatic moieties in the modern
468 feathers, although their precise assignment to dedicated compounds is uncertain. In the ^{13}C NMR
469 spectrum, the 129 ppm peak is typical for aromatic carbons, including those from phenylalanine and
470 tyrosine (Yoshimizu & Ando 1990; Jain *et al.* 2015). Finally, the peak at 158 ppm can be ascribed to the
471 O–alkyl C of tyrosine and/or the C of the guanidino group ($\text{N}-\text{C}=\text{N}$) of arginine (Yoshimizu & Ando

1990; Jain *et al.* 2015). This spectrum is in agreement with previous reports indicating that keratin is a major constituent of feathers (Lucas & Stettenheim 1972). The ^{13}C NMR and IR spectra of the modern feathers also shares some similarities with various types of melanins (Ito and Nicol 1974; Duff *et al.* 1988; Adhyaru *et al.* 2003; Centeno & Shamir 2008).

The much simpler ^{13}C NMR spectra of the fossil feathers and their surrounding sediment are dominated by long alkyl chains with a low contribution of aromatic carbons. This is also consistent with the IR spectra (Fig. 7B), mainly showing contributions of aliphatics and silicate and carbonate minerals. When compared to the ^{13}C spectrum of the modern feathers, the aliphatic signal in the fossil is poorly resolved; the aromatic peak is broad and no resonance signal could be detected in the carboxylic region. These features indicate that the proteinaceous contribution identified in the buzzard feathers is no longer present in the fossil sample. The comparison between the FTIR spectrum of the modern feather and that of the fossil feathers (Fig.7A) shows that the characteristic bands of amides are absent in the IR spectrum of the fossil feathers. However, a more precise comparison can be achieved at the molecular level thanks to pyrolysis in the presence of TMAH coupled with GC-MS.

Molecular building blocks of organic matter

TMAH Py-GC-MS analysis of modern feathers thus highlights the presence of glycine, serine, leucine, alanine, valine and proline moieties in buzzard feather keratin, in agreement with previous studies on feather keratin (Fig. 8A; O'Donnell & Inglis 1974; Arai *et al.* 1983, 1986; Murphy *et al.* 1990; Staroń *et al.* 2011; Saravanan & Dhurai 2012). Additionally, pyrolysis products derived from isoleucine, phenylalanine and tyrosine occurred in substantial amounts although they are often considered as minor constituents of feather keratin. However, homolysis of the side chain of phenylalanine and tyrosine

493 favours high yields in TMAH pyrolysis (Gallois *et al.* 2007). Despite its acknowledged high abundance
494 in feather keratin, cysteine is absent in the pyrochromatogram of buzzard feathers, probably because it
495 mainly releases H₂S upon pyrolysis (Moldoveanu 2009), not detected in the presently used analytical
496 conditions. Alternatively, some of the identified products (methylbenzene **1**, methyl, methoxybenzene **9**)
497 as well as glycine derivatives may originate from melanin, although they are poorly diagnostic
498 compounds (Stępień *et al.* 2009). However, the melanin signal was reported to be overwhelmed by
499 protein-derived products upon pyrolysis of bulk feathers (Barden *et al.* 2011).

500 The pyrochromatograms of the fossil feathers and their ‘host’ sediment are comparable as they are both
501 dominated by series of alkane/alkene doublets and fatty acid methyl esters as well as minor compounds
502 eluting at the beginning of the pyrochromatogram. The latter include a methoxybenzene substituted by
503 two methyl groups or an ethyl group **22** which originates from lignin or polysaccharides such as
504 cellulose, depending on its substitution pattern (Seitz & Ram 2000; Choi *et al.* 2013). Despite these
505 similarities, differences are observed, including the much weaker abundance of octadecanol **24** in the
506 sediment pyrolysate. These differences clearly show that even though some imprint from the sediment
507 may have contributed to the fossil feather pyrolysate, at least some features are typical for the fossil
508 feathers. They notably include the C₁₈ doublet and octadecanol **24**. The predominance of the
509 alkane/alkene doublets in the pyrolysate is in agreement with the strong aliphatic signal observed in
510 NMR and FTIR (bands at 2690, 2920 and 2851 cm⁻¹) (Figs 6, 7). A similar highly aliphatic character
511 has been reported in Eocene bird feathers (O’Reilly *et al.* 2017) but also in soft tissues from other fossil
512 organisms, such as in cuticles from Carboniferous arthropods (Baas *et al.* 1995; Stankiewicz *et al.*
513 1998), skin from a Cretaceous mummified hadrosaur (Manning *et al.* 2009) and Cretaceous fish scales

514 (Gupta *et al.* 2008). C₁ to C₃ alkylbenzenes were also identified in pyrolysates of Oligocene weevil and
515 tadpole, and associated matrix (Gupta *et al.* 2007; Barden *et al.* 2015).

516 The aliphatic series dominating the pyrolysate of the fossil feathers reflect either selective preservation
517 of macromolecular aliphatic matter pre-existing in the extant organism (Tegelaar *et al.* 1989) or *in situ*
518 polymerization of aliphatic lipids (Stankiewicz *et al.* 2000; Gupta *et al.* 2007). No such aliphatic series
519 could be detected in the pyrolysate of modern feathers, probably precluding the first hypothesis. In
520 contrast, several aliphatic series were identified in the lipid extract of the modern feathers (*n*-alkanes, *n*-
521 acids, *n*-alcohols; data not shown). Indeed, modern bird feathers are coated by lipids as protection
522 against adverse environmental factors. Such lipids, secreted by the uropygial gland, were shown to be
523 preserved through geopolymerisation in an Eocene bird (O'Reilly *et al.* 2017). A similar
524 geopolymerisation can be put forward to account for the occurrence of aliphatic moieties of the present
525 fossil feathers. Endogenous lipids may be transformed into more stable geopolymers, composed of
526 alkane/alkene doublets, and can therefore be 'preserved' and traced in vertebrate fossils (O'Reilly *et al.*
527 2017).

528 The absence of signals typical for proteinaceous material in the NMR and IR spectra and
529 pyrochromatogram of the fossil feathers is noteworthy. It is further by the weak N/C ratio in the fossil
530 when compared with modern feathers. The lack of proteinaceous components consistent with keratin
531 was previously suggested on the same fossil based on TOF-SIMS and IR analyses (Lindgren *et al.*
532 2015). In agreement with the commonly accepted lability of proteins, this feature suggests their
533 extensive degradation in our specimen upon diagenesis. Recent taphonomy experiments on extant
534 feathers demonstrated substantial degradation of keratin upon microbial and thermal decay (Saitta *et al.*

2017). Moreover, it must be noted that diagenetic degradation of proteinaceous moieties was previously put forward for Eocene birds (O'Reilly *et al.* 2017; Saitta *et al.* 2017) and Palaeozoic annelid fossils (Dutta *et al.* 2010). However, even if no proteinaceous compounds were detected in *Anchiornis* feathers, one cannot exclude the possibility of finding similar biomolecules in other fossils in the future.

Although PIXE analyses showed that C and S are closely associated in the fossil feathers, no organosulphur compound could be detected in the pyrolysate. Altogether, the lack of organosulphur compounds (such as thiophenes) in the fossil feather pyrolysates and the lack of C=S/C–S species in their IR spectrum strongly suggest a lack of organic-S species in this sample. In contrast, S incorporation was evidenced through FTIR and TMAH-Py-GC-MS in melanosomes of Miocene frogs, thus demonstrating involvement of natural sulphurization in the preservation of the fossil organic matter (McNamara *et al.* 2016). The lack of organosulphur compounds in *Anchiornis* pyrolysate should thus reflect diagenetic conditions that prevented such natural sulphurization of organic matter. In the sedimentary environment, the sulphurization of organic matter to form organosulphur compounds requires the presence of reactive organic matter and inorganic sulphides (i.e. anoxic conditions), with sufficient, but not excessive, reactive iron. If reactive iron exceeds a certain quantity, iron sulphides (pyrite) would precipitate instead (Canfield 1989; Werne *et al.* 2000). Here, our results suggest that the concentration of S and Fe in the environment was high enough to form iron sulphides (i.e. pyrite framboids and microcrystallites). The occurrence of iron oxides or hydroxides as framboid crystals (SEM and EDX characterization) suggests that S may have been preferentially used for the formation of iron sulphides (such as pyrite) during early diagenesis (Sinninghe-Damsté & De Leeuw 1990). During later diagenesis, pyrite framboids were probably *in situ* weathered into the iron oxides and hydroxides observed beneath the carbonaceous surface of feathers, thus releasing S that may have been further

557 associated with organic compounds, e.g. through the formation of chelate with melanin (Wogelius *et al.*
558 2011). Such associations may have favoured/enhanced organic matter preservation and are consistent
559 with the interrelation between S and C highlighted by PIXE analyses.

560 Recently, a mechanism of nitrogen preservation based on lipoxidation and glycooxidation of protein was
561 proposed in biomineralized tissues of diverse Mesozoic and Cenozoic vertebrates, fossilized in oxidative
562 settings (Wiemann *et al.* 2018). Such a process, which can be catalysed by transition metal such as iron,
563 may have led to the preservation of the small amount of nitrogen in YFGP-T5199. However, the present
564 study deals with soft tissues and not biomineralized one. Additionally, so far this preservation
565 mechanism could only be evidenced in oxidizing environment (Wiemann *et al.* 2018). The occurrence of
566 iron oxide framboids in the studied *Anchiornis* fossil probably resulting from pyrite weathering, rather
567 attests for the reducing conditions of fossilization, thus making the involvement of this preservation
568 pathway unlikely (i.e. lipoxidation).

569 Further experimental studies on modern feathers and comparisons with the fossil record are required to
570 explain why keratin is not preserved in *Anchiornis* feathers although melanin has been detected and
571 melanosomes and moulds have been observed.

572

573 **CONCLUSIONS**

574

575 The methods used in this study provide new and complementary information on how the plumage of
576 *Anchiornis huxleyi* (YFGP-T5199) is preserved. SEM and EDS reveal that fossil feathers are preserved
577 in a fine-grained material constituted of K-rich phyllosilicates, illite and interstratified illite/smectite.

PIXE analyses show that both light (C, N, O) and heavy (S, Na, Ca, etc.) elements are present in the fossil samples, even at very low concentrations. The presence of iron oxide pseudomorphs after pyrite likely indicates a reduced depositional environment for *Anchiornis*. Carbon is the dominant element in the fossil feathers, which are also enriched in sulphur with respect to the ‘host’ sediment. EBS mapping of the interior of the samples revealed a decrease in carbon concentration with depth. Our analysis therefore shows that the fossil feathers are preserved in the uppermost part of the sample, as a thin (*c.* 3 μm -thick) S-bearing carbonaceous layer. High-resolution imaging of the feather microstructure revealed the presence of elongated microbodies (650–950 nm), probably corresponding to eumelanosomes. Molecular characterization of the organic matter in the ‘host’ sediment, fossil feathers and modern feathers by ^{13}C -NMR, micro-ATR FTIR and Py-GC-MS shows that the fossil does not display the complex amino-acid signature typical for keratin, the main constituent of modern feathers. Although the organic matter of the fossil feathers and their ‘host’ sediment are both dominated by aliphatic moieties, they exhibit substantial differences (distribution pattern of series, occurrence of components specific to the feathers) suggesting that the organic matter of the fossil feathers is derived, at least partially, from original constituents of the feathers.

Altogether, these results show that the fossil feathers can be described as compression fossils, as described in Schweitzer (2011, p. 192). The finely grained (clay-rich) host sediment contributed to the morphological preservation of *Anchiornis* soft tissues. As stressed by Schweitzer (2011, p. 192), the very fine grain size of the sediments might have prevented the degradation of soft tissues by microbes, and subsequent loss of degraded organic matter in the environment before and during diagenesis. However, the lack of protein-derived moieties in the fossil organic matter shows that the latter has been significantly altered during diagenesis. The excellent morphological preservation of the fossil soft tissue

600 is not associated here with a high preservation level of organic matter. Hence, the fossil feathers have
601 likely undergone a complex diagenetic history including several steps affecting differentially their
602 morphology and chemistry. *In situ* polymerization of lipids into more stable aliphatic compounds during
603 early diagenesis was likely the main process responsible for organic matter preservation in the fossil
604 feathers. Additionally, sulphur was probably involved in several steps of the fossil preservation although
605 no natural sulphurization took place.

606 Our results are therefore unique by combining different analytical techniques on Jurassic fossil feathers.
607 This integrative multidisciplinary study appears as a powerful approach to decipher morphological,
608 mineralogical, structural and chemical features of fossil soft tissues and their fossilization processes.
609 This study provides new insights into the taphonomy of labile compounds, suggesting that keratin,
610 unlike the pigment melanin, is not present in the feathers of *Anchiornis huxleyi* (YFGP-T5199). Further
611 analyses of fossil feathers of different ages and depositional settings are required to better understand the
612 preservation potential of melanin and keratin.

613 Finally, we used here for the first time on a Jurassic fossil Ion Beam Analysis (IBA), a non-destructive
614 analytical technique providing an in-depth profiling of C to U elements. Further developments of this
615 technique to palaeontological samples might help at identifying the precise location of fossil soft tissues
616 within the sediment and then characterizing metal elements that are directly associated with the
617 fossilized tissues.

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626

627 **DATA ARCHIVING STATEMENT**

628 Additional data for this study are available in the Dryad Digital Repository:

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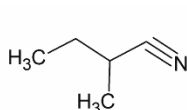
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APPENDIX 1

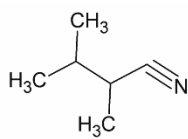
Molecular structures of compounds identified in the modern bird feathers; numbers correspond to those appearing in Table 2 and Figure 8.



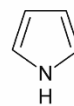
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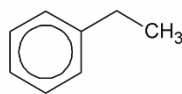
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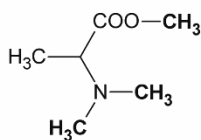
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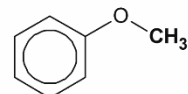
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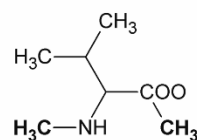
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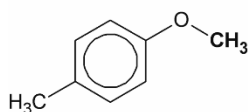
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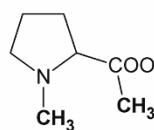
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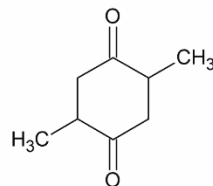
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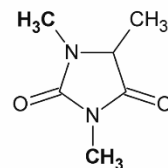
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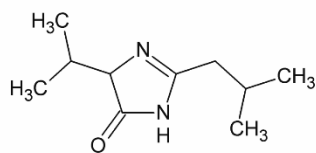
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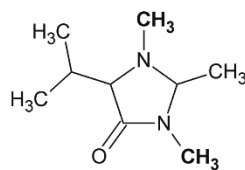
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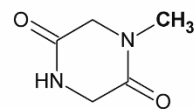
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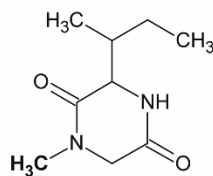
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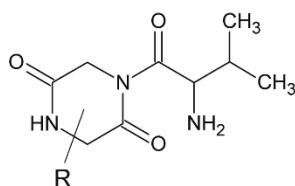
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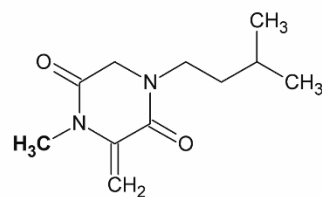
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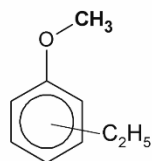
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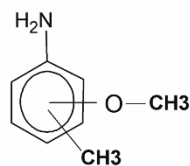
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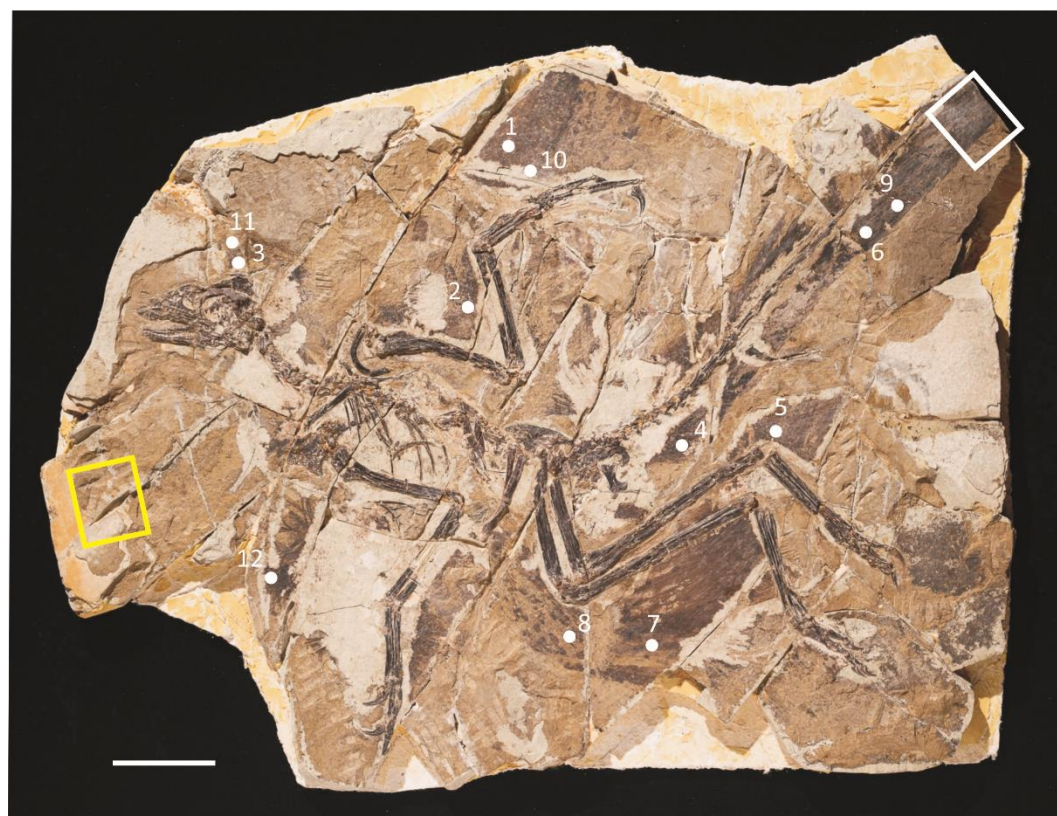
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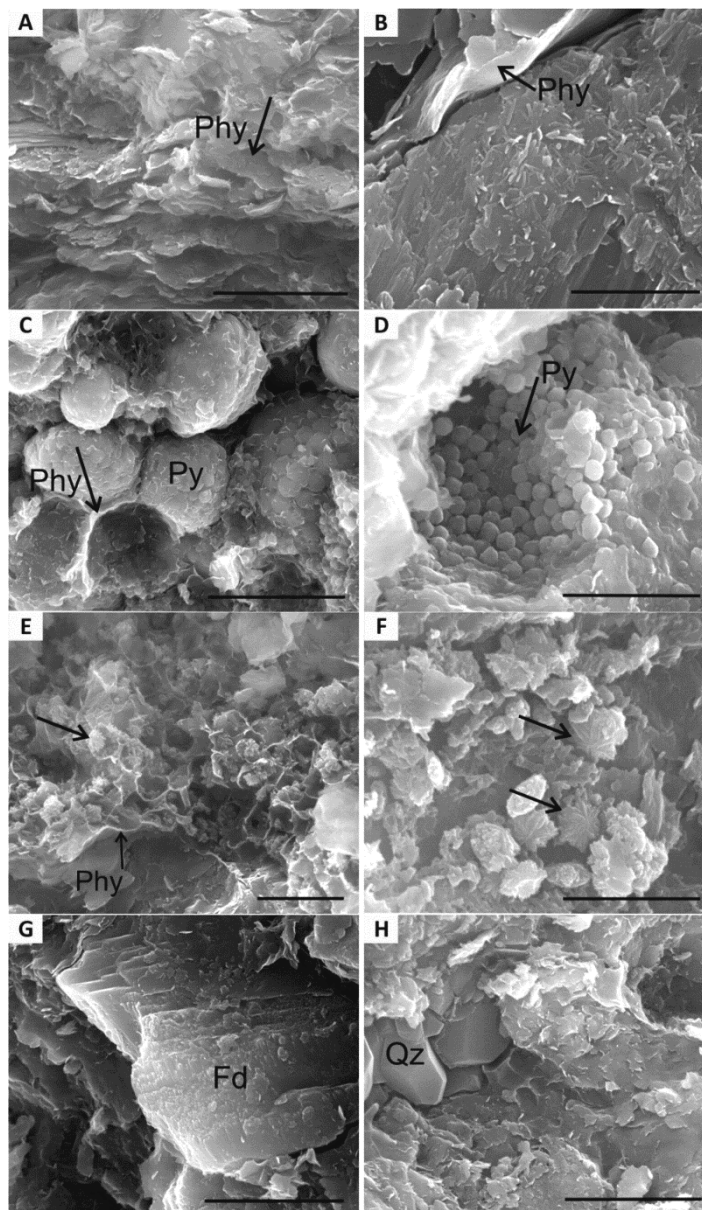
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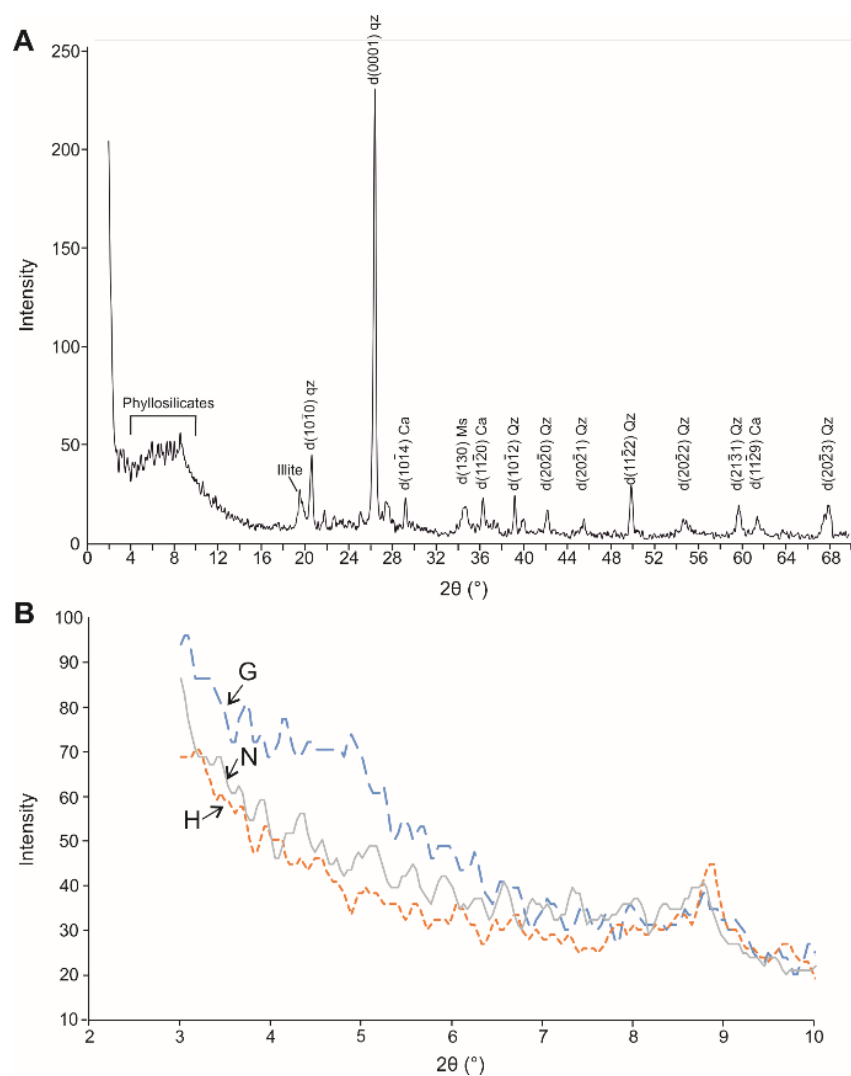
1043 **FIG. 1.** *Anchiornis huxleyi* (YFGP-T5199). Photograph of the Jurassic feathered theropod with location
1044 of sampled areas. The white box (upper right) indicates locations of fossil feather and ‘host’ sediment
1045 sampling, while the yellow box (lower left) indicates location of ‘remote’ sediment sampling, for NMR,
1046 Py-GC-MS, and IBA analyses. White dots are samples used for SEM imaging and EDS. Scale bar
1047 represents 5 cm. Photograph by Thierry Hubin (RBINS). Colour online.
1048



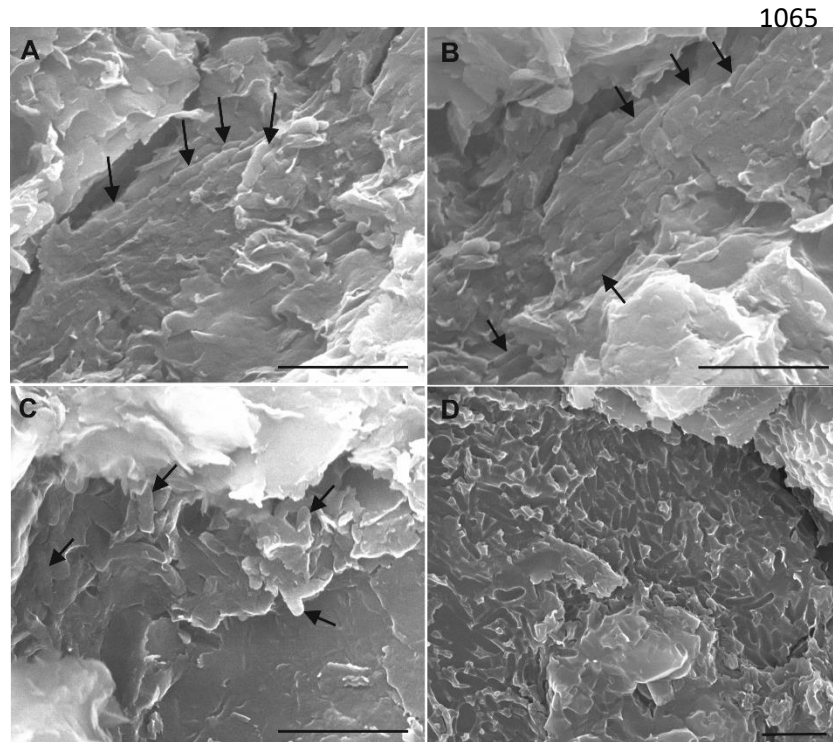
1049 **FIG. 2.** Scanning electron microscopy (SEM) images of minerals observed in the plumage of
 1050 *Anchiornis huxleyi*. A–B, thin platelets of phyllosilicates observed in sample 7 (see fig. 1 for location).
 1051 C, pyrite framboids (sample 1). D, pyrite crystallites (sample 1). E, clayed pores containing small star-
 1052 shaped iron-oxide crystals (sample 8). F, star-shaped iron oxides (sample 1). G, feldspar crystal
 1053 surrounded by clay sheets (sample 5). H, quartz crystals embedded in a clayed matrix (sample 9).
 1054 Arrowheads indicate the aforementioned minerals. Abbreviations: Fd, feldspar; Phy, phyllosilicate; Py,
 1055 pyrite; Qz, quartz. Scale bars represent: 5 μm (A, D, G, H); 3 μm (B); 10 μm (C); 2 μm (E, F).
 1056



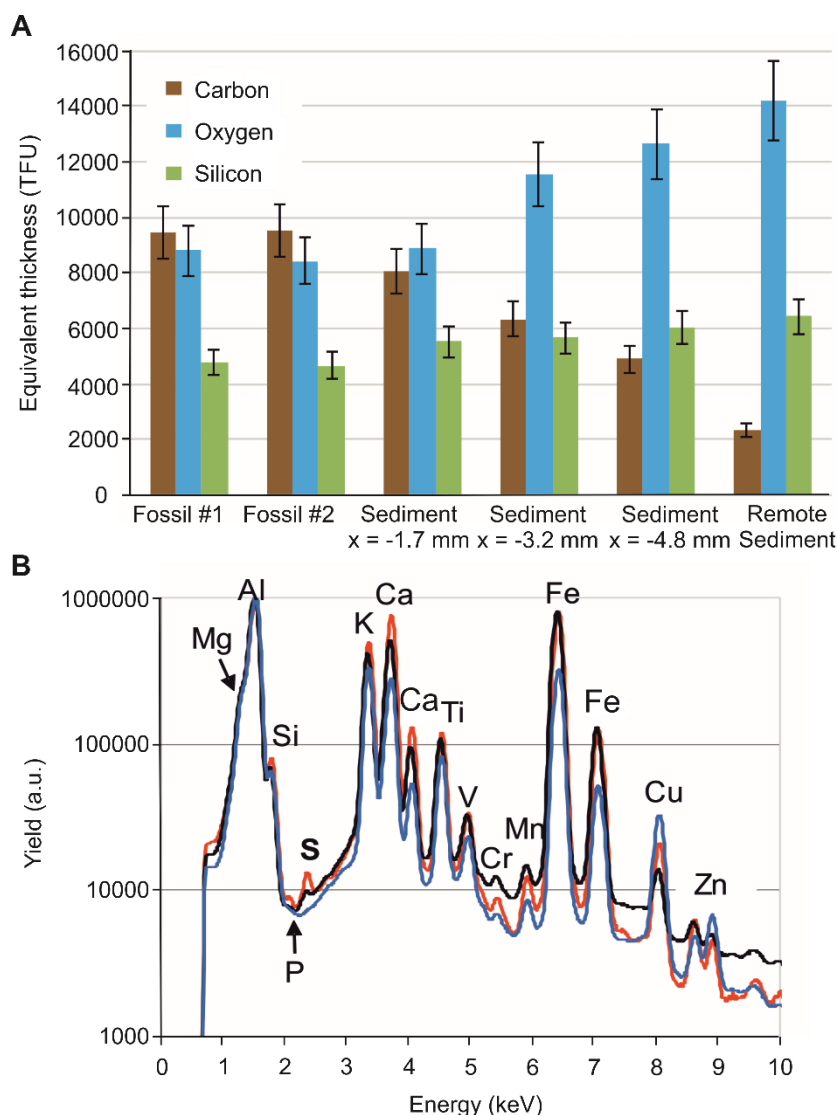
1057 **FIG. 3.** X-ray powder diffraction patterns of the ‘host’ sediment. A, spectrum of the bulk rock; ca,
 1058 calcite; qz, quartz. B, the fraction <2 μm , with spectra of natural (N), glycolated (G), and heated (H)
 1059 sample; the expansive illite/smectite interstratifications can be observed in the spectrum of the
 1060 glycolated (G) material through shifts in the position of the peaks. Colour online.



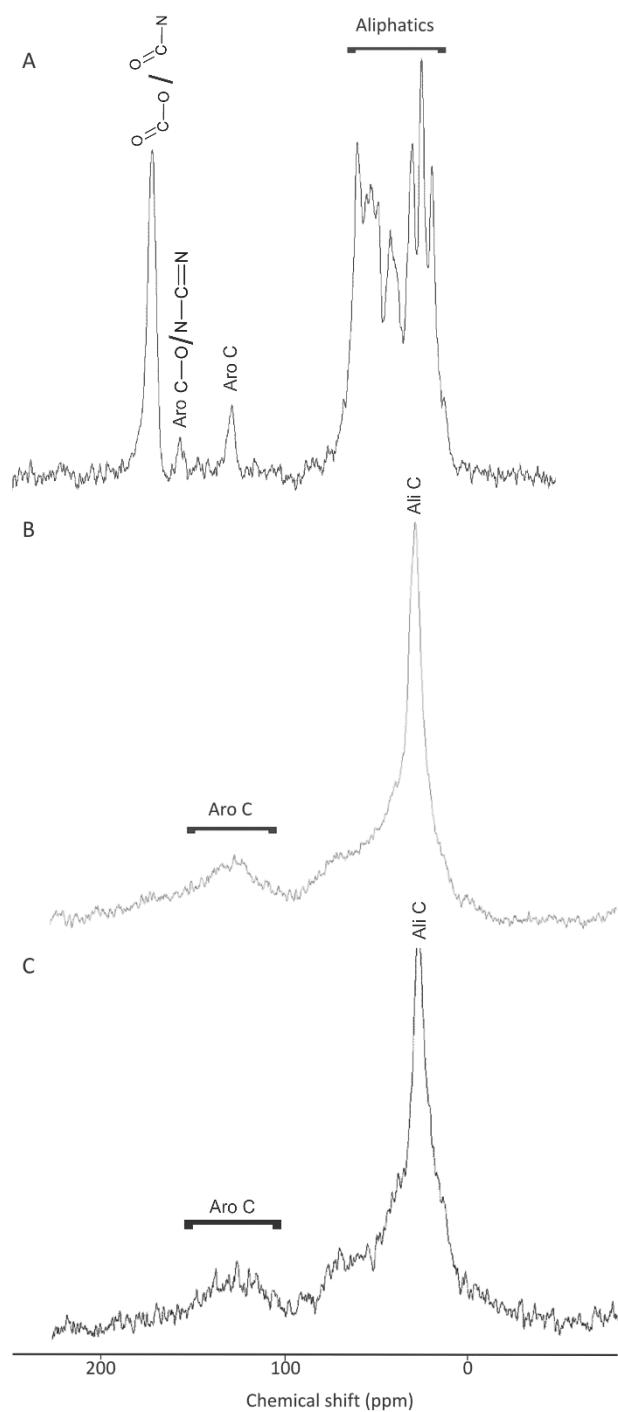
1061 **FIG. 4.** SEM images of the ultrastructure of *Anchiornis* plumage. A–B, elongated microbodies (arrows)
1062 observed in sample 6 (see Fig. 1 for location). C, elongated microbodies (arrows) observed in sample 9.
1063 D, imprints observed in samples 6. All scale bars represent 2 μm .
1064



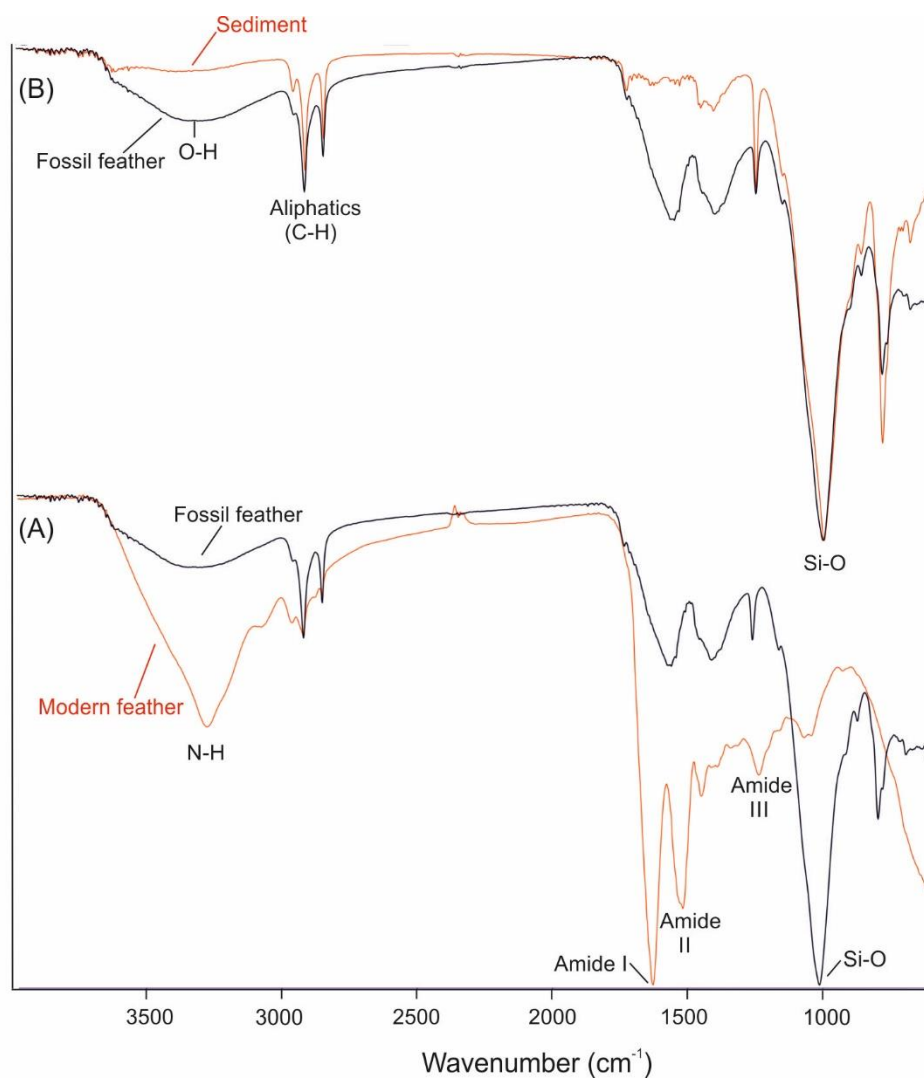
1066 **FIG. 5.** Results of elastic backscattering spectrometry (EBS) and particle-induced x-ray emission
 1067 (PIXE) analyses on the fossil feathers, the 'host' sediment and the 'remote' sediment. A, global content
 1068 of C, O and Si obtained by EBS, in integrating the C, O and Si depth profiles over the 0–25 000 TFU
 1069 interval; error bars give an estimate of the uncertainties considering the counting statistics as well as the
 1070 cross-section and stopping power uncertainties; analysis was performed in duplicate on the same area of
 1071 the fossil feather (Fossil #1, Fossil #2), three points of analysis were taken at three different locations in
 1072 the 'host' sediment (at 1.7, 3.2 and 4.8 mm from the fossil), and one in the remote sediment.
 1073 B, PIXE spectra obtained for one point of analysis in the fossil feather (red), the 'host' sediment (black),
 1074 and the remote sediment (blue); the spectra are normalized to the Al signal (which comes mainly from
 1075 the selective filter) to allow for a direct comparison.



1076 **FIG. 6.** Cross polarization/magic angle spinning ^{13}C nuclear magnetic resonance (CP-MAS ^{13}C NMR)
 1077 spectra of: A, the modern bird feather; B, the fossil feathers; C, the 'host' sediment. Major chemical
 1078 functions are indicated on each peak.
 1079



1081 **FIG. 7.** IR spectra showing the comparison of: A, a modern buzzard feather with *Anchiornis* feathers; B,
 1082 *Anchiornis* feathers with the sedimentary matrix ('remote' sediment). See the text for peak assignment.
 1083 The spectra are normalized to 100% transmittance. Colour online.



1084 **FIG. 8.** Chromatograms of the products formed during the pyrolysis of: A, modern buzzard feathers; B,
 1085 fossil feathers; C, remote sediment. Peak identifications are given in Table 2. C_n, carbon chain, with n
 1086 indicating the length of the chain.
 1087

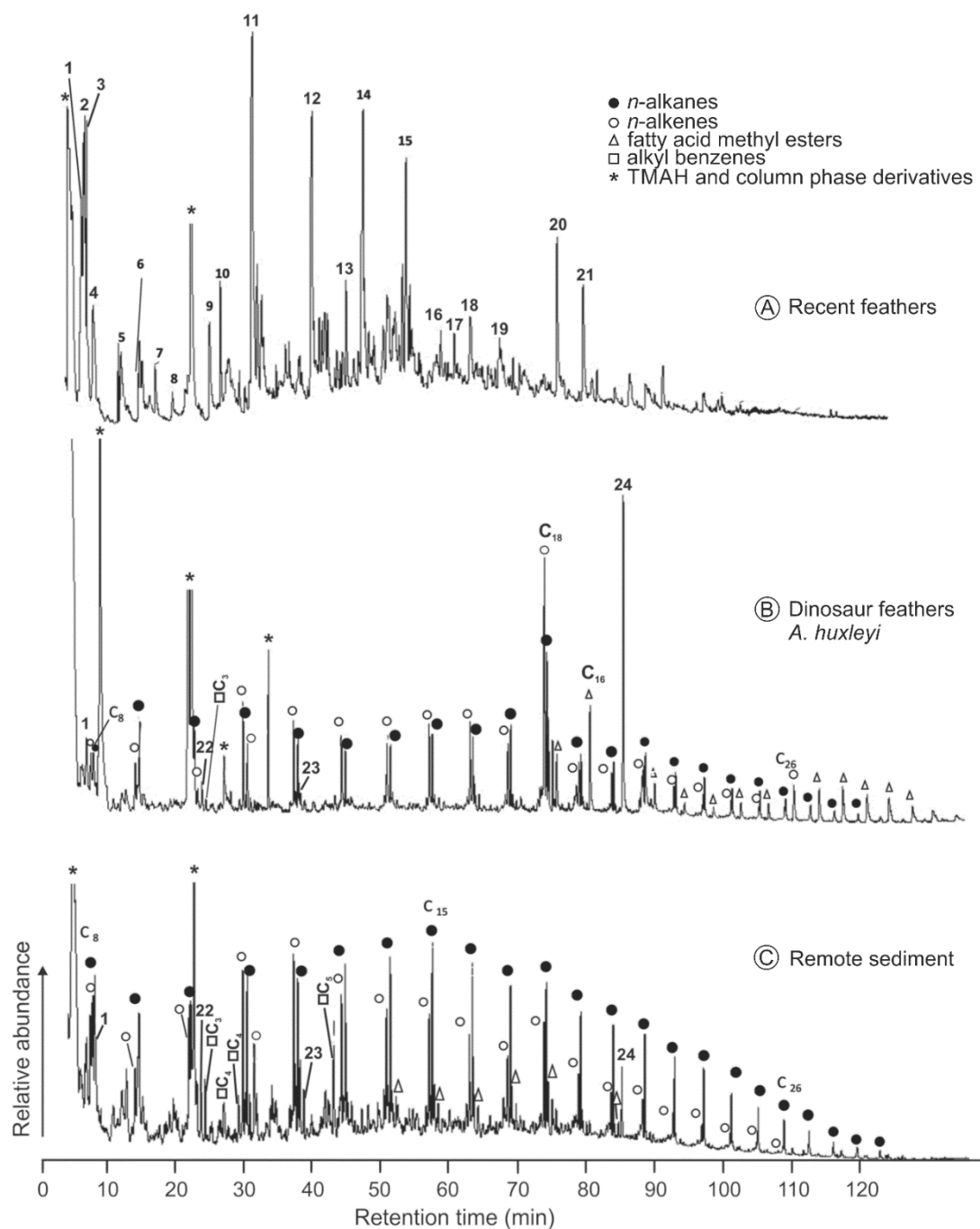


Table 1. PIXE derived concentration data for S and C in the fossil and modern feathers, and “host” sediment.

	[S] (ppm)	[C] (ppm)
Fossil Feather #1	1739	80,385
Fossil Feather #2	1946	82,171
+1.7 mm*	1162	72,012
+3.2 mm*	801	49,213
+4.8 mm*	893	41,835
Remote sediment	98	20,331
Rachis	37,142	547,174
Barbs	43,070	314,962

*distance of the analysed spot from the feather/sediment boundary.

See Cincotta et al. (2020, table 1) for details on the global uncertainty calculations, Fig. 1 and Cincotta et al. (2020, fig. S2) for the location of the analyses.

Peak	Retention time (min)	Major characteristic ions ^a (m/z)	Molecular ion	Compound	Possible origin ^b	Modern feather	Fossil feather ^c	Embedding sediment ^c
1	6.7	<u>91</u> ; 92; 39; 65	92	Methylbenzene	(Phe)	X	X	X
2	6.9	<u>55</u> , 54, 42	83	2-methylbutanenitrile	Ileu	X		
3	7.1	<u>43</u> , 41, 39, 68	83	3-methylbutanenitrile	Leu	X		
4	8.0	<u>67</u> ; 41; 39; 40	67	1 <i>H</i> -Pyrrole	(Ser)	X		
5	12.1	<u>91</u> ; 55; 106; 65	106	Ethylbenzene	(Phe)	X		
6	14.8	<u>72</u> ; 42; 56; 131	131	N,N- Dimethylalanine methylester	Ala	X		
7	17.0	<u>108</u> ; 78; 65; 39	108	Methoxybenzene	Tyr	X		
8	19.5	<u>86</u> ; 102; 42; 55	145	N-Methyl-valine Methyl Ester	Val	X		
9	25.0	<u>122</u> ; 77; 107; 91	122	1-Methoxy-4-methylbenzene	Tyr	X		
10	26.5	<u>84</u> ; 42; 100; 58	143	N-Methyl-proline Methyl Ester	Pro	X		
11	31.2	<u>56</u> ; 140; 42; 112; 83	140	2,5-Dimethylcyclohexane-1,4-Dione	Gly	X		
12	39.7	<u>42</u> ; 127; 142; 56	142	1,3,5-Trimethylimidazolidine-2,4-Dione	Ala?	X		
13	44.8	<u>56</u> ; 126; 139; 41	182	1-Isobutyl-4-isopropylimidazolinone	Val	X		
14	47.2	<u>128</u> ; 42; 71; 113	170	5-Isopropyl-1,2,3-trimethylimidazolidinone	Val-(Gly)	X		
15	53.6	<u>128</u> ; 42; 57; 71	128	1-Methylpiperazine-2,5-dione	Gly	X		
16	58.6	<u>82</u> ; 167; 182; 110	182	?	His?	X		
17	60.5	<u>152</u> ; 41; 55; 137; 179	194	?	Val?	X		
18	62.9	<u>142</u> ; 113; 42; 71	198	1-Methyl-3-(1-methylpropyl)piperazine-2,5-dione	Ileu-Gly	X		
19	67.1	<u>142</u> ; 113; 42; 98; 212	?	N-(1-oxo-2-amino-3-methyl-butyl)piperazine-2,5-dione derivative	Val	X		
20	75.5	<u>139</u> ; 70; 42; 168	210	N-Methyl-3-methylidene-6-(3-methylbutyl)piperazine-2,5-dione	Ser-Leu	X		
21	79.2	<u>168</u> ; 139; 70; 42	210	Isomer of compound 20	Ser-Leu	X		
22	23.7	<u>136</u> , 121, 122, 91	136	1-Methoxy-2,3-dimethylbenzene or 1-Ethyl-2-methoxybenzene	Lignin or cellulose		X	X
23	38.1	<u>136</u> , 122, 137	137	Methoxy-methylaniline	?		X	X
24	86.3	<u>45</u> ; 57; 97; 224; 252	284	1-Methoxyoctadecane	?		X	X
●		<u>43</u> ; 57; 71; 85		<i>n</i> -alkanes	Aliphatic chains		C ₈ -C ₃₀ (C ₁₈ , C ₁₁)	C ₈ -C ₂₉ (C ₁₅)
○		<u>55</u> ; 43; 69; 83, 97		<i>n</i> -alk-1-enes	Aliphatic chains		C ₈ -C ₂₆ (C ₁₁ , C ₁₈)	C ₈ -C ₂₉ (C ₁₁ , C ₁₈)
Δ		<u>87</u> ; 74; 43; 55		Fatty acid methyl esters	Aliphatic chains		C ₈ -C ₃₀ (C ₁₆)	C ₈ -C ₃₀ (C ₁₆)
□		91, 105		Alkyl benzenes	?		C ₃	C ₃ -C ₅ (C ₅)

1094 Table 2. Main products released from pyrolysis of modern feathers, fossil feathers and embedding sediment in the presence of TMAH.

1095 ^aMS fragments are in order of decreasing abundance, with base peak underlined.
1096 ^bCompounds in brackets indicate possible origin that is not univocal; Ala, alanine; Gly, glycine; His, histidine; Ileu, isoleucine; Leu,
1097 leucine; Phe, phenylalanine; Pro, proline; Ser, serine; Tyr, tyrosine; Val, valine; ? tentative origin.
1098 ^cC_{range} (C_{max}, C_{submax}).

1099

1100