The animal origin of thirteenth-century uterine vellum revealed using non-invasive peptide fingerprinting

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Tissue-thin parchment made it possible to produce the first pocket Bibles, thousands of which were made in the thirteenth century. The source of this parchment, so-called 'uterine vellum,' is one of the longstanding controversies in codicology. Use of the Latin term abortivum in many sources has led some scholars to suggest that the membranes were made from fetal calf or sheep. Others have argued that it would not be possible to sustain herds if so many pocket Bibles were produced from fetal skins, arguing instead for exotic alternatives such as rabbit. Here we report a simple and objective technique using standard conservation treatments to identify the animal origin of all parchment. The non-invasive method is a variant on ZooMS peptide mass fingerprinting but extracts protein from the parchment surface by using electrostatic charge generated by gentle rubbing of a PVC eraser on the membrane surface. Using this method we analysed 72 pocket Bibles originating in France, England and Italy, and 256 further parchment samples which bracket this period. We find no evidence of exotic animals, but do find the use of more than one mammal species in a single manuscript, consistent with the local availability of hides. These results suggest that ultrafine vellum does not necessarily represent the use of abortive or newborn animals with ultra-thin hides, but could equally well derive from a production process that allowed the skins of maturing animals of several species to be rendered into vellum of equal quality and fineness.

pocket Bible | parchment | vellum | collagen | mass spectrometry

Introduction

One of the outstanding controversies in the field of codicology concerns the origin and production of so-called uterine vellum. William Horman, writing in 1519, uses the term 'abortyue' to refer to the rendered hide of a fetal animal. This material is called 'abortive,' he claims, 'bycause the beest was scante parfecte' in the uterus (1). Latin phrases such as *charta virginea* and *pergamena virginea* appear regularly in medieval and early modern sources alongside the formulation *charta non nata* (literally 'unborn sheet'), strongly implying a material made from the skin of an aborted or miscarried foetus.

Those in the field of manuscript studies have long disputed the derivation of this ultra-fine writing material. Some older scholarship suggested that uterine vellum probably derived from the hides of smaller, more thin-skinned mammals: thus Pollard spoke of what he believed were 'probably rabbit-skin duodecimos' (2), while Thompson (3) suggested that 'some of what now masquerades as "uterine vellum"' may actually be rabbit or squirrel parchment.' Most palaeographers continue to view the idea of medieval uterine vellum as myth or superstition, believing that its production on a large scale would have entailed a prohibitively high number of aborted foetuses. As Christopher de Hamel puts it in a definitive statement, "*it is very difficult to believe that thousands of cows miscarried for generations, or were deprived of their foetuses in such numbers to supply the booktrade economically….If the term uterine parchment must be used at all, it should perhaps refer to a quality of skin and not to its origin*" (4). Other proposed solutions to the derivation of this material have involved specific production processes; Clarkson, for example, argues that uterine vellum may have entailed the splitting of skins (5). The importance of "*Proper analysis of parchment species*" and more specifically the origin of uterine vellum has also been highlighted as a priority research question by Mark Clarke who states "*The question of 'uterine vellum' versus the use of small animals would be one historic debate it would be well to resolve once and for all, and would provide useful localisation data*" (6).

The scholarly disputes notwithstanding, uterine vellum must represent either (i) the selection of specific animals whose skin was uniquely fine or (ii) the development of craft skills to work a wide range of skins into ultra-fine sheets--or, of course, both at once. In order to explore this question we examined the animal composition of ultra-fine parchment (which in our selected samples ranged from 0.03 - 0.28 mm), sampled primarily from pocket-sized codices, and compared these samples with other

Significance

The study reports the first use of triboelectric extraction of protein from parchment. The method is non-invasive and requires no specialist equipment or storage. Consequently samples can be collected without need to transport the artifacts; instead researchers can sample when and where is appropriate and analyse when required. Extracted proteins from 466 parchment samples are used to resolve the long-standing question of the origin of 'uterine vellum'. We find no evidence of exotic species, such as rabbit or squirrel. We suggest that 'uterine vellum' was often an achievement of technological production using available resources, and would not have demanded unsustainable agricultural practices. This conclusion has wide implications for the study of medieval manuscripts by scholars in disciplines across the humanities.

Reserved for Publication Footnotes

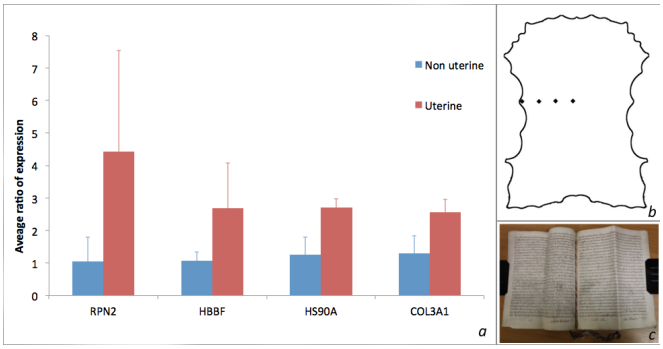

Fig. 1. |iTRAQ experiment. a) Comparison of protein expression of uterine and non-uterine parchment samples. Uterine samples show higher expression levels for 4 proteins including Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2 (RPN2), Hemoglobin fetal subunit beta (HBBF), Heat shock protein HSP 90-alpha (HSP90A) and Collagen alpha-1(III) chain (COL3A1). Error of 2 standard deviations b) Uterine calfskin indicating the four sampling points. c) Historical legal document dated to 1776 used as one of the non-uterine samples.

parchments which bookend the main period of production. If the skins of small animals (rabbits, squirrels) were used, their presence would be revealed by ZooMS. If uterine vellum was sourced from uniquely fine skins of domestic animals we might suspect a predominance of calfskin as implied by the etymology of the word *vellum*, though medieval terminology is notoriously inconsistent in this regard. Indeed it has been suggested that the term 'vellum' itself may well have been used to refer to the quality of the writing surface as much as to the calf it came from. If the production of uterine vellum represented instead a specialized craft skill and available skins could be worked to produce ultrafine parchment sheets, then the selection of animals would be similar to that evident in other coarser membranes from the same geographic region.

The most frequently cited examples of uterine vellum are the thirteenth-century Paris Bibles (2, 3, 5, 7, 8). These books marked an important transition in the history of the medieval Vulgate Bible. They were one-volume Bibles (pandects) with a consistent organization, which meant that they were easily searchable, making them the ideal reference guide for study in the case of students and for preparing sermons in the case of the clergy (9). One of the most important subgroups of thirteenth-century Paris Bibles is the pocket or portable Bible, volumes sufficiently small to be easily transported. Ruzzier (10) suggests that the total output of portable Bibles in the thirteenth century could have exceeded 20,000 copies. The majority (54%) were made in France, most in Paris (10), although Bibles of this style were also produced in England at the same time, and slightly later in both Italy (notably in the Veneto) and Spain.

Species Identification of Parchment

In order to assess the origin of uterine vellum, the principal line of evidence is the identification of the skin. Skins have been identified by overall size, thickness, colour, levels of grease and patterns of follicles (11). Ryder used follicle pattern (12) to identify species but not all parchments had discernable follicle patterns left, nor could every pattern be identified. Protein (13, 14) and DNA based methods (15–19) potentially offer absolute determination of species. Toniolo et al. (13) analysed 5 mg of parchment from the thirteenth-century 'Marco Polo Bible' at the Biblioteca Medicea Laurenziana, and used peptide sequences to identify a single leaf as calfskin. Kirby et al. (14) also used destructive sampling techniques to analyse a Quran and other objects, "*a few 10's of micrograms...as small a sample as can be handled with a fine tweezers ...is sufficient to produce satisfactory results*". Teasdale et al. (15) have recently reported genomic data from post-medieval parchment using, on average, 50 mg of parchment.

The challenge for destructive sampling methods, such as those outlined above, is finding sufficient appropriate samples. Recently a collection of more than 100 sacrificial historic parchment samples (1480 - 1965) has been made available for analysis (20). However, many scholars wish to explore specific documents; Toniolo et al. (13) admit that the remaining leaves of the 'Marco Polo's Bible' (which could not be subjected to destructive analyses) may have been from sheep - as indeed were all three of the family's archival documents that we have analysed.

A further hidden cost of molecular analysis is adequate storage of extracted samples. It is usually necessary to freeze DNA and protein extracts to preserve the integrity of the sample. In the case of libraries and archives, this would add a further cost burden either in shipping or storage.

Spectroscopic methods are increasingly being used (21), but despite the advent of mobile laboratories (e.g. MOLAB 22), the high costs of the analytical devices mean they are few in number and not readily accessible to every library and archive. Furthermore, spectroscopic methods cannot discriminate between species, thus we do not know if any of the differences observed (e.g. lipid content) are due to production, deterioration, historical conservation or merely species differences between hides.

Triboelectric extraction

Here we describe a novel non-invasive molecular identification method, using electrostatic molecular extraction onto a solid phase polyvinylchloride (PVC) polymer. PVC polymer erasers are widely accepted by the conservation community as a non-invasive measure for removing dirt. As a consequence every archive and conservation studio has access to and experience of the use of PVC erasers. A further advantage of the extraction method is that protein is preserved on the PVC polymer waste at room temperature with no further storage requirements (apparently indefinitely). It can therefore be retained at point of collection without special preparation or storage until it is deemed appropriate by the researcher for a set of samples to be analysed. The method is easily scalable and places the sampling in the hands of curators, codicologists and conservators. Using this approach we have sent kits (consisting of PVC erasers, nitrile gloves, and sampling tubes) to 14 archives and 40 libraries. They have returned for analysis 79 thirteenth-century pandect Bibles, including 72 pocket Bibles.

Results and Discussion

In order to assess the quality of our results we performed a comparative analysis of the same sample using conventional ZooMS techniques that require destructive sampling (14) as well as non-destructive eZooMS (electrostatic ZooMS). For the purpose of this experiment we used two different documents: (a) a sixteenth-century Manorial Court Roll and (b) an eighteenth-century seal tag. In case (a) a fragment of parchment of approximately 0.5 x 0.5cm was used, and in case (b) a fragment of approximately 0.1cm x 0.3cm was used. In both cases an eZooMS sample was taken from the main document.

The optimisation of our eZooMS methodology has allowed us to obtain equal, if not better, results than when using an actual fragment of parchment (Fig 1 supplementary). The observed difference in quality is probably due to an excessive amount of collagen being extracted during destructive analyses (Fig 2 supplementary). However, the eraser technique itself may also contribute to giving a cleaner signal by retaining contaminating molecules which interfere with the subsequent analysis on the PVC.

Uterine Skins?

We explored the use of LC-MS/MS to see if differential biomarkers for uterine skin could be identified. Four samples

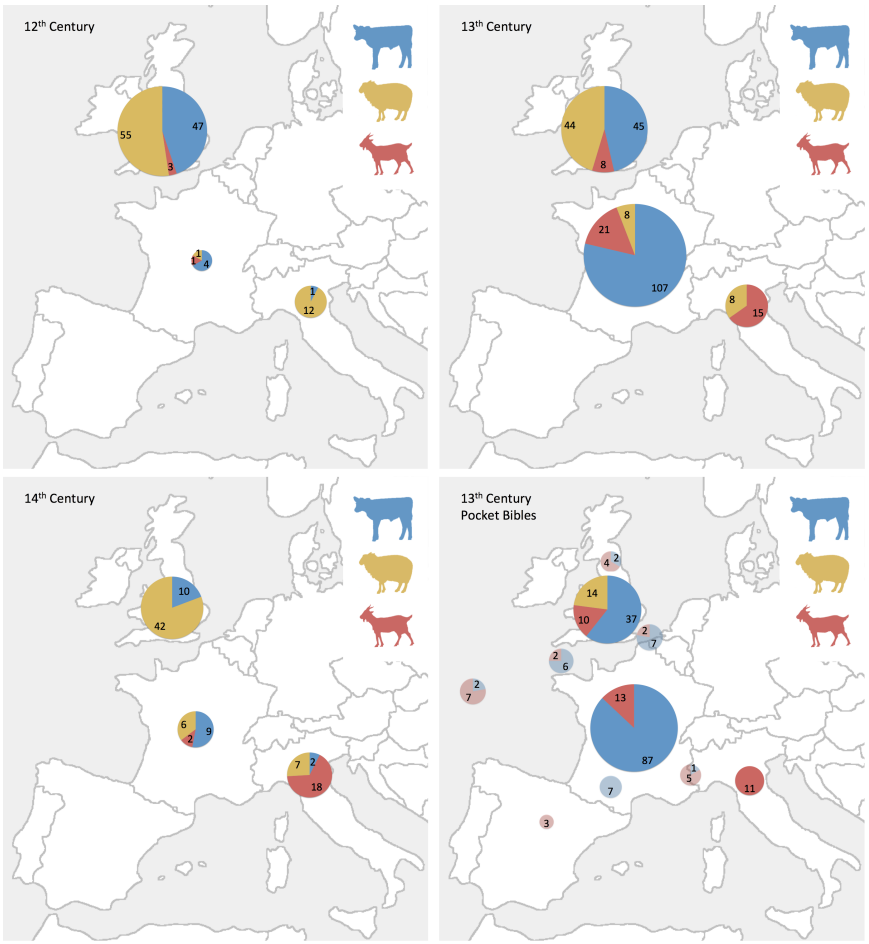

Fig. 2. The relative proportions of animals used to make parchment in each of the three regions studied (France, England and Italy) during the twelfth to fourteenth centuries. The size of the circle indicates the number of samples. Except for the figure describing exclusively pocket Bibles, data was obtained from all sources of parchment including legal documents, secular codices, and Bibles using the eZooMS method.

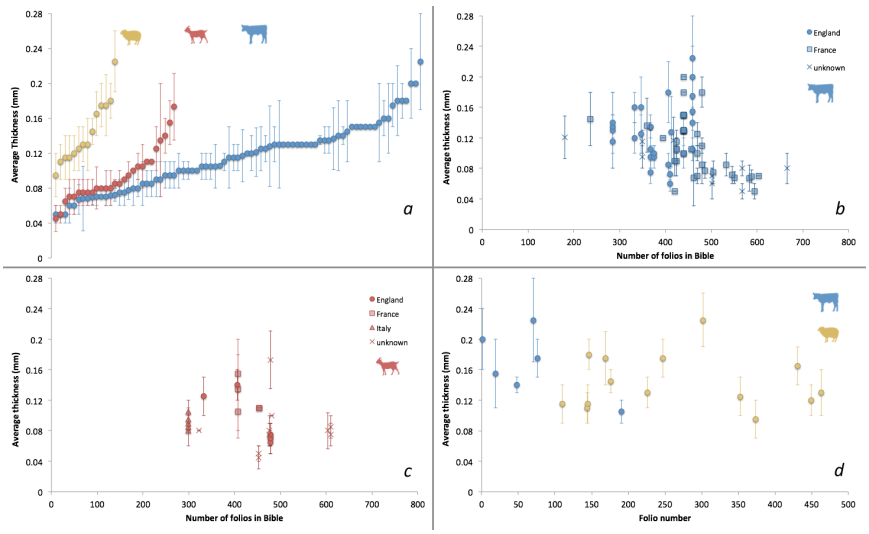

Fig. 3. Thickness of folios. a) Thickness of all pocket Bible folios by species. b) Thickness of calf pocket Bible folios by total number of folios in Bible. c) Thickness of goat pocket Bible folios by total number of folios in Bible. d) Thickness of folios from pocket Bible Ee.6.26.

were taken from a modern uterine calfskin and compared to four non-uterine calf skins (two modern samples and two historic samples dated to 1776). iTRAQ labeling was used on both sets of samples in order to determine quantitative differences between uterine and non-uterine parchment (Fig 1). Uterine samples showed elevated expression of four proteins: Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2, Hemoglobin fetal subunit beta, Heat shock protein HSP 90-alpha and Collagen alpha-1(III) chain. Unfortunately, none of the peptide markers observed in eZooMS mapped to any of these proteins, so it is not possible to report the presence of these proteins in our Bible samples. The elevated expression levels of fetal haemoglobin and collagen III are consistent with previous studies (23, 24). Brandt et al (25) identified calfskin leather from

Neolithic capes from Denmark based upon the presence of fetal calf haemoglobin in one sample. However, fetal haemoglobin clears after eight to ten weeks in cattle (24, 26) and six to eight weeks in sheep and goat (24, 27), preventing distinction between fetal and calf hides.

Species variation

Species identifications of all the samples analysed are included in Table 1S and 2S (supplementary information). From the 72 pocket Bibles sampled a total of 220 folia were analysed. Of these 68% (149 folia) were calf, 26% (57 folia) were goat and 6% (14 folia) were sheep. We find no evidence for the rabbit skin duodecimos suggested by Pollard (2). Of the 72 Bibles we sampled, 62 were sampled on multiple pages. In most cases the identification was consistent for each of the folia of one manuscript, including the 18 leaves of the Hornby Bible (OSU.MS.MR.Frag.74). However, there were at least five Bibles composed of parchment from multiple species. In order to confirm the presence of multiple animals we were able to analyse 20 folia of one of these, Cambridge University Library Ee.6.26 six of which were identified as calf and 14 as sheep. The species distinction is mirrored by stylistic differences within the Bible which suggest that this manuscript may in fact be a composite of two different Bibles. The first part of the Bible (fol. 1-108) resembles a "proto-'Paris' Bible" model produced ca. 1200-30 whereas the second part (fol. 109-459) is a far better fit for the "mature 'Paris' Bible" blueprint of ca. 1230-80 as described by Laura Light (9, 28–30). It is also worth noting that the attributed English provenance relates to inscriptions present in the second part of the Bible; no provenance indicators are recorded for the first part, so it is possible that the Bible's first 108 folia could have been produced in France.

Geographic spread of animal exploitation

Previous authors have identified geographic variation in the animals used for parchment. Forbes (31) citing Wattenbach notes that hides used for parchment were predominantly calf in the north of Europe and sheep and goat in the south. Ruzzier (10) agrees, and suggests that to the north of the Alps fine parchment was probably made from calfskins. Clarkson (5) noting the "warm, creamy tint" and "flexibility" of late-medieval Italian skins attributes these qualities to the use of goatskin and possibly alternative methods of preparation. De Hamel (32) anticipates that Italian parchment is often prepared from goatskin, which contrasts with the the poor representation of goat in England (33, 34), see also (35).

In order to examine whether the geographic variation observed for ultra-fine parchment was different from that of coarser membranes we surveyed a further 256 parchment objects from the eleventh to fourteenth centuries. The selection of skins appears to reflect available livestock, and therefore a city's or a region's preferences - sheep are most abundant in England, calf in France and goat in Italy. In Italy there is an absence of goat in the eleventh century (contra 36) which possibly reflects regional variation. Six (of 12) eleventh-century Italian sheep samples were sourced from Sicily, a sheep-based dairying economy. Whilst there are differences between the two centuries spanning the period of interest, any study of secular changes will require a more comprehensive investigation.

The pattern of selection of ultra-fine parchment for Bible production seems to be broadly in line with other parchment analysed (Fig 2). The English Bibles seem to be more varied in their construction, including multiple species in the same volume. Of the five Bibles that were composed of a mixture of animals (calf and goat or calf and sheep) three of them have an English provenance, one a possible English provenance and the fifth a French or Italian provenance. Of the seven larger Paris Bibles (i.e. classified as Non-pocket pandect Bibles) the three described as being of French production, as well as the two Bibles from the Low Countries and Germany, were all identified as calf.

Medieval literary production in general (37) and parchment production in particular is linked to agriculture, more specifically to meat consumption (e.g. 16). Parchment production was presumably located close to the point of slaughter, because skins deteriorate within days resulting in poor quality, spotty membranes (38). Hides can be preserved by salting, but in Northern Europe this would be prohibitively expensive: the salt price in England rose from a base above 0.1g Ag kg^{-1} during this period. Goat horncore accumulations are not unusual, particularly in the eastern part of the country and in urban sites. This has led to the suggestion that they may be the product of an international skin trade, as horns were routinely left within the transported skins (33).

The presence of goatskin in the English pocket Bibles is intriguing, an assertion that is further supported for one sample with DNA evidence (S3). Goatskin folia produced in England are thinner than their Italian or French counterparts. This would seem to indicate that goatskin was not routinely available and may have been acquired specifically for the production of pocket Bibles.

If we exclude horncores, in both Saxon and medieval England sheep are overwhelmingly more common than goats, the latter invariably representing less than 10% of the sheep/goat total, and often this proportion is close to 1%. Goat occurrence is mentioned at about 50% of sites, but invariably in small frequency. In late medieval times goat further declined as attested by both documentary (39) and archaeological (40) sources.

A survey of English archival documents (Fiddyment unpublished data) reveal that nearly all are written on sheepskin parchment (one power of attorney from Wales 1342, is written on goat). This selection of sheepskin parchment for legal documents may not merely be one of cost. The *Dialogus de Scaccario* (41) argues for the use of sheepskins as the medium for English legal documents due to the difficulty of erasure of text as a result of the propensity of sheepskin parchment to delaminate. This propensity means that sheep is also the easiest skin to split, apparently making it ideally suitable for the thin parchment used in pocket Bibles. However, we have found only one example of a Pocket Bible made of sheepskin: this is of supposed English provenance.

Two larger Paris Bibles of English origin were also found to be made from sheep. The pocket Bible (Cambridge University Library Ee.6.26) is made up of both calf *and* sheep parchment. Five of the six folia identified as calf parchment were located in the first part of the book (folia 1-108) thought to be written at an earlier date (pre-1230). The 14 folia made of sheepskin (and one of calfskin) parchment were identified in the second part of the Bible (folio 109 onwards) which is dated slightly later (post 1230).

At Italian archaeological sites, sheep bones also tend to predominate for the medieval period, but far less so than in England, with a typical sheep:goat ratio ranging between 3:1 and 2:1 (42–44). Italian reports, however, rarely provide specific identifications for caprines. The predominance of goat horncores is not a phenomenon known for Italian sites.

Cattle were predominantly slaughtered as adults in medieval England, which reflects their major role as traction force. The gradual replacement of working cattle with horses towards the end of the period – at least in some regions – provided the opportunity for an increase in early culling. From the Fifteenth century onwards several sites, mainly urban, have produced relatively high proportions of calf bones, probably a consequence of an increased demand for veal and milk, as well as a greater production of calfskins (40, 45).

In Italy the use of cattle is clearly variable, with emphasis on meat production or traction, according to the site. The presence

of very young animals is, however, reported at several urban sites (44) or at high status sites (46). The nature of the current evidence is insufficiently consistent, or thoroughly reported, to identify any clear chronological trend. Excavations in multiple sites from the Parisian area revealed a sample of 3102 bones identified as either cattle (30.4%), caprines (38.2%) or swine all dating from the thirteenth century (47). Clavel notes that in the Thirteenth and Fourteenth centuries, calf bones are rare in the collections, making up only 2 to 5% of all cattle bones from the sites, becoming much more frequent in later periods. Veal calves were typically slaughtered at around six months, but according to La Lande, writing in the mid eighteenth century (48) vellum should be prepared from much younger calfskins (less than two months). Practice bears this out, cattle hides rapidly becoming too thick to work into parchment, however the bones of very young cattle are rare in the Middle ages (40, 47).

Our results have shown that pocket Bibles, some of the first examples of commercial book production (9), were written on all three species used widely in parchment production. In our survey of 220 folia spanning three centuries as reported in this study, no exotic species were recorded. The use of sheep in only one of 72 pocket Bibles indicates that it was not favoured for these very thin membranes despite that fact that sheepskin delaminates and that the range of thicknesses measured for calf and sheep parchment are similar (0.09-0.28mm for calf and 0.07-0.26 for sheep) (Fig 3). The presence of goatskin and sheepskin parchment in the sample set would seem to indicate that uterine calfskin was not necessary to produce very fine membranes.

Fourteenth century accounts from Beaulieu Abbey (49) show calfskin was more highly valued than sheepskin. The importance of the skin as a source of revenue is repeatedly evidenced by the abundance of flay marks on animal remains, the range of decrees and bylaws restricting the flaying of animals that had died of diseases, and the lengths the authorities went to in order to prevent these skins being used (50). The intact skeleton of a cow from a fourteenth century burial at Téteghem Carlines 3 (North France) that died of a dystocic calving (51) gives a further insight into the importance (or otherwise) of uterine hides. Whilst the cow's hide has been removed, despite the fact that it could have been easily released and flayed to obtain uterine calfskin for parchment, the calf remains trapped and unflayed in the birth canal.

Conclusion

The study reveals the value of the triboelectric eZooMS approach to the analysis of parchment. It requires no specialist equipment or storage;samples can be collected when appropriate without the need to transport the artifacts; and they can be analysed when required. As we have shown (Fig 1 supplementary), triboelectric extraction is more efficient than physical sampling for four reasons. Firstly, much less material is required. Secondly, the molecular extraction is bulked onto a large volume of eraser waste which means it is easy to sub-sample. Thirdly, molecules are stabilised on the surface of the eraser waste. Fourthly, eZooMS acts as a pre-purification step, as evidenced by the results from whole samples. Following extraction, the pigmented extracts remain on the eraser crumbs: only the macromolecules are extracted.

We have been able to provide the first significant molecular evidence to resolve the long-standing question of the origin of *Pergameno velym*. We find no evidence of exotic species, such as rabbit or squirrel. Whilst in France this parchment did derive mostly from calfskin, in other places different skins were used subject to local availability. The production of ultra-thin parchment was the result of a technological production process using available resources and, as such, would not have demanded unsustainable agricultural practices. We have further shown that manuscript document made from animal skins are a valuable additional resource of archaeologists exploring patterns of consumption.

Although use of uterine vellum cannot be discounted, our results suggest that it is not a defining factor of production. Instead, our findings would seem to emphasize dependence on a highly specialised craft technique rather than the supply of a particular raw material. A more likely explanation for the production of fine parchment is the use of relatively young animals and the deployment of pouncing techniques using pumice which enabled the corium to be ground to the desired thickness. The density of collagen fibrils in calf and goat, compared to a more open weave and higher fat content in sheep parchment, favours the former two species; nevertheless it is evident that parchment makers had the skills to make fine parchment from all three.

Materials and Methods

All codices sampled were classified in their catalogue entries as thirteenth-century Bibles.The 79 Bibles were subdivided, depending on their dimensions, into two groups based on criteria used by Ruzzier: Pocket Bibles (height+width < 385 cm) and Non-pocket Bibles (height+width >385 cm). Details of all Bibles sampled can be found in Tables 1S and 2S (supplementary information).

Sampling

Samples were extracted in the participating archives and libraries using kits sent by SF consisting of 1.5ml microcentrifuge tubes, nitrile gloves, acid free paper, and non-abrasive conservator's erasers. Sampling was performed using a Staedtler 'Mars Plastic' eraser , rubbing the eraser in one direction and collecting the resulting eraser waste fragments in individual 1.5ml microcentrifuge tubes. For each sample a new individual piece of eraser and acid free paper was used and thrown away once sampling of the folio was completed to avoid cross-contamination. Nitrile gloves were worn throughout the sampling process to avoid keratin (human skin) contamination. Sample collection was undertaken on areas of the document that had no writing and presented structural integrity (absence of holes or tears in the parchment). All samples were stored at room temperature until required, usually by the partner. Details of each document sampled were entered onto an online spreadsheet shared between the partner and the laboratories in York.

eZooMS

Initially eraser crumb samples were spun down at maximum speed on a benchtop centrifuge for one minute and 75μl of 0.05M NH_3CO_3 (AmBic) buffer (pH 8) was added to each sample. Samples were heated at 65°C for 1 hour. Once cooled, 1μl of trypsin [0.4μg/μl] was added and samples were incubated at 37°C for 18 hours. However, at a later stage the method of collagen extraction was optimised by removing the heating step and condensing the process into one incubation step at 37°C for 4 hours with both AmBic and trypsin added simultaneously. After incubation with trypsin, digests were spun down at maximum speed on a benchtop centrifuge for one minute and 1μl of 5% TFA was added. Samples were desalted and concentrated using C18 resin (Millipore, Durham, UK), following the manufacturer's instructions. Peptides were eluted in a final volume of 50ul of 50% acetonitrile/0.1% TFA (v/v). 1ul of eluted peptides was mixed on a ground steel plate with 1μl of α-cyano-4-hydroxycinnamic acid matrix solution (1% in 50% ACN / 0.1% TFA (v/v/v)) and air dried. All samples were spotted in triplicate. Samples were analysed using a calibrated Ultraflex III [NLD1] (Bruker Daltonics, Bremen, DE) MALDI-TOF instrument in reflector mode. Spectral analysis was performed using the open-source cross-platform software mMass (www.mmass.org; 52) and individual peptides were identified manually according to Buckley et al (53, 54).

iTRAQ

Four samples of uterine calfskin each measuring 0.5x0.25cm were prepared as well as four control 'non-uterine' calf skins also measuring 0.5x0.25cm. Samples were incubated in 0.5M TEAB, pH 8.5 at 65°C for one hour in order to solubilise the collagen. The supernatant was quantified using a Qubit. 150 μg of protein from each sample was digested overnight with trypsin at 37 °C at a ratio of 1:75, trypsin to protein. The tryptic digest was transferred over C18 resin to desalt and concentrate peptides by washing with 0.1% trifluoroacetic acid (TFA). Peptides were eluted in a final volume of 50 μl of 50% acetonitrile (ACN)/0.1% TFA (v/v). Digested samples were labeled with the iTRAQ reagents following the protocol provided by the vendor (Applied Biosystems, Foster City, CA). Briefly, one vial of iTRAQ labeling reagent was used for every 100 μg of protein. Ethanol was used to solubilize the iTRAQ reagent then added to the peptide sample ensuring a final organic concentration of at least 60% (v/v). The labeling reaction was performed by 2 h incubation at room temperature. Samples were cleaned up prior to LC-MS/MS analysis using a cation-exchange cartridge system (ICAT) and desalted using a C18 cartridge.

Dried peptides were resuspended in 2% ACN and 0.1% TFA and analyzed by nano–liquid chromatography tandem mass spectrometry (nano–LC-MS/MS) as described previously (55). In brief, samples were separated using a nanoUPLC (Easy spray C18 column with a 75 μm × 500 mm, 2.1 μm particle

size; Thermo-Fisher) coupled to a Q Exactive tandem mass spectrometer (Thermo Scientific, Bremen, Germany). MS data was acquired with a resolution of 70,000 at m/z 200 and selecting the Top 15 precursor ions. Ion target in MS1 was $3x10^6$ and $5x10^5$ in MS2 mode. Ions with m/z between 380 and 1800 were accumulated for up to 100ms in MS1 and 128ms in MS2. We used a stepped normalized collision energy at 31, 34 and 37%, a precursor isolation window of 1.6 m/z with an offset of 0.3 m/z, a fixed first mass of 100 m/z and a resolution of 35000 (profile) for MS2 spectrum acquisition. The samples were loaded in 0.1% TFA in 1% CH_3CN and eluted with a gradient of 3% -35% CH_3CN in 5% DMSO and 0.1% formic acid in 60 minutes at a flow rate of 250 nl/minute.

Identification and quantitation of iTRAQ signals was processed with PEAKS 7 (Bioinformatics Solutions Inc). Raw files were refined to correct precursor mass and MS2 spectra searched against the Uniprot reference proteome of bos taurus (04/07/2014). Search parameters include trypsin with up to 2 missed cleavage sites, 10ppm mass tolerance for the precursor and 0.05Da mass tolerance for fragment masses. Carbamidomethylation was a fixed modification while we used the PTM search module to identify otherwise modified peptides. For quantitation of the 8-plex iTRAQ reporter signals we used the default setting of the quantitation module.

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1. Horman W (1519) *Vulgaria Viri Doctissimi Guil. Hormani* (Richard Pynson, London).
2. Pollard G (1941) Notes on the Size of the Sheet. *Library* 4(2-3):105–137.
3. Thompson D (1936) *The materials of medieval painting* (bcin.ca).
4. De Hamel C, Museum B (1992) *Scribes and Illuminators* (University of Toronto Press).
5. Clarkson C (1992) Rediscovering parchment: the nature of the beast. *Pap Conserv* 16(1):5–26.
6. Clarke M (2014) The Archaeology of the Book: Formulating Analytical Research Questions. *e-conservation Journal* 2:10–16.
7. Ruzzier C (2013) The Miniaturisation of Bible Manuscripts in the Thirteenth Century. A Comparative Study. *Form and Function in the Late Medieval Bible*, eds Light L, Poleg E (Brill Academic Publishers), pp 105–125.
8. Dove M (2012) *The Blackwell companion to the Bible and culture* ed Sawyer JFA (John Wiley & Sons).
9. Light L (2011) The Bible and the individual: The thirteenth century Paris Bible. *The Practice of the Bible in the Middle Ages: Production, Reception, and Performance in Western Christianity*, eds Boynton S, Reilly DJ, pp 228–246.
10. Ruzzier C (2013) The miniaturization of Bible manuscripts in the XIIIth century. A comparative study. *Form and Function in the Late Medieval Bible*, eds Poleg E, & Light L (Brill), pp 105–125.
11. Juchauld F, Bonnenberger P, Komenda A (2010) Identification de l'espèce animale des cuirs de reliure et des parchemins. *Matériaux Du Livre Médiéval*, Bibliologia., ed M. Zerdoun CB (Turnhout Brepols Publishers), pp 13–28.
12. Ryder ML (1960) Parchment—its history, manufacture and composition. *J Soc Arch* 2(9):391–399.
13. Toniolo L, D'Amato A, Saccenti R, Gulotta D, Righetti PG (2012) The Silk Road, Marco Polo, a Bible and its proteome: a detective story. *J Proteomics* 75(11):3365–3373.
14. Kirby DP, Buckley M, Promise E, Trauger SA, Holdcraft TR (2013) Identification of collagen-based materials in cultural heritage. *Analyst* 138(17):4849–4858.
15. Teasdale MD, et al. (2015) Paging through history: parchment as a reservoir of ancient DNA for next generation sequencing. *Philos Trans R Soc Lond B Biol Sci* 370(1660). Available at: http://dx.doi.org/10.1098/rstb.2013.0379.
16. Stinson T (2011) Counting Sheep: Potential Applications of DNA Analysis to the Study of Medieval Parchment Production. *Kodikologie Und Paläographie Im Digitalen Zeitalter 2 - Codicology and Palaeography in the Digital Age 2*, Schriften des Instituts für Dokumentologie und Editorik — Band 3., eds Franz F, Fritze C, Vogeler G (Books on Demand (BoD), Norderstedt), pp 191–207.
17. Bower MA, Campana MG, Checkley-Scott C, Knight B, Howe CJ (2010) The potential for extraction and exploitation of DNA from parchment: a review of the opportunities and hurdles. *J Amer Inst Conserv* 33(1):1–11.
18. Campana MG, et al. (2010) A flock of sheep, goats and cattle: ancient DNA analysis reveals complexities of historical parchment manufacture. *J Archaeol Sci* 37(6):1317–1325.
19. Burger J, Hummel S, Herrmann B (2000) Palaeogenetics and cultural heritage. Species determination and STR-genotyping from ancient DNA in art and artefacts. *Thermochim Acta* 365(1–2):141–146.
20. Možir A, Cigić IK, Marinšek M, Strlič M (2014) Material properties of historic parchment: A reference collection survey. *Stud Conserv* 59(3):136–149.
21. Bicchieri M, Monti M, Piantanida G, Pinzari F, Sodo A (2011) Non-destructive spectroscopic characterization of parchment documents. *Vib Spectrosc* 55(2):267–272.
22. Brunetti BG, Matteini M, Miliani C, Pezzati L, Pinna D (2007) MOLAB, a Mobile Laboratory for In Situ Non-Invasive Studies in Arts and Archaeology. *Lasers in the Conservation of Artworks*, Springer proceedings in physics. (Springer Berlin Heidelberg), pp 453–460.
23. Mays PK, Bishop JE, Laurent GJ (1988) Age-related changes in the proportion of types I and III collagen. *Mech Ageing Dev* 45(3):203–212.
24. Kitchen H, Brett I (1974) Embryonic and fetal hemoglobin in animals. *Ann N Y Acad Sci* 241(0):653–671.
25. Brandt LØ, et al. (2014) Species Identification of Archaeological Skin Objects from Danish Bogs: Comparison between Mass Spectrometry-Based Peptide Sequencing and Microscopy-Based Methods. *PLoS One* 9(9):e106875.
26. Grimes RM, Duncan CW, Lassiter CA (1958) Bovine fetal hemoglobin. I. Postnatal persistence and relation to adult hemoglobins. *J Dairy Sci* 41(11):1527–1533.
27. Breathnach CS (1964) Foetal and neonatal haemoglobins in sheep and goats. *Q J Exp Physiol Cogn Med Sci* 49:277–289.
28. Light L (1984) Versions et révisions du texte biblique. *trad de l'anglais), dans Bible de tous les temps* 4:55–93.
29. Light L (1994) *The Early Medieval Bible. Its Production, Decoration, and Use* ed Gameson R (Cambridge University Press).
30. Light L, Marsden R, Matter EA (2012) The thirteenth century and the Paris Bible. *The New Cambridge History of the Bible*, eds Marsden R, Matter EA (Cambridge University Press, Cambridge), pp 380–391.
31. Forbes RJ (1955) *Fibres and Fabrics of Antiquity: Washing, Bleaching, Fulling and Felting; Dyes and Dyeing; Spinning, Sewing, Basketry and Weaving; Weaving and Looms; Fabrics and Weavers* (Brill).
32. De Hamel C, Museum B (1992) *Scribes and Illuminators* (University of Toronto Press).
33. Albarella U (2003) Tanners, tawyers, horn working and the mystery of the missing goat'. *The Environmental Archaeology of Industry Oxford: Oxbow Books*:71–86.
34. Pascua E (2009) From Forest to Farm and Town: Domestic Animals from ca. 1000 to ca. 1450. *A Cultural History of Animals in the Medieval Age*, ed Resl B (Berg 3PL).
35. Magrini S (2007) Production and Use of Latin Bible Manuscripts in Italy during the Thirteenth and Fourteenth Centuries. *Manuscripta* 51(2):209–257.
36. Bianchi F, et al. (1993) Facteurs de variations de l'épaisseur du parchemin italien du VIII Eau XV Esiècle. *Ancient and Medieval Book Materials and Techniques (Erice, 18-25 September 1992)*, Studi e Testi ., eds Maniaci M, Paola Munafò PF (Vatican City), pp 95–184.
37. Hanna R (2005) *London Literature, 1300-1380* (Cambridge University Press).
38. Thompson D (1956) *The Materials and Techniques of Medieval Painting* (Dover Publications).
39. Dyer C (2004) Alternative agriculture: goats in medieval England. Available at: https://lra.le.ac.uk/handle/2381/8911.
40. Albarella U (1997) Size, power, wool and veal: zooarchaeological evidence for late medieval innovations. *Environment and Subsistence in Medieval Europe*, ed De Boe G & Verhaeghe (Institute for the Archaeological Heritage of Flanders, Bruges), pp 19–30.
41. (Ernest Flagg) HE The Dialogue Concerning the Exchequer. circa 1180. Available at: http://www.yale.edu/lawweb/avalon/medieval/excheq.asp.
42. Colonnelli G, De Grossi Mazzorin J (2000) Nuovi dati sll'alimentazione a Farnese (VT) nei secoli XV e XVI. *In Atti Del Secondo Convegno Nazionale Di Archeozoologia*, ed Edizioni A, pp 369–376.
43. Minniti C (2009) Economia e alimentazione nel Lazio medievale: nuovi dati dalle evidenze archeozoologiche. *Archeologia medievale* 36:275–283.
44. Corbino C (2009) "Dall'allevamento alle mense" la Toscana tra il XIII e la prima metà del XV secolo. Analisi archeozoologiche. PhD diss. (Universita degli Studi di Siena).
45. Albarella U (2005) Meat production and consumption in town and country. *Town and Country in the Middle Ages: Contrasts, Contacts and Interactions*:1100–1500.
46. Baker P, Clark G (1993) Archaeozoological evidence for medieval Italy: a critical review of the present state of research. *Archeologia medievale* (20):45–78.
47. Clavel B (2001) L'animal dans l'alimentation médiévale et moderne en France du Nord (XIIIe-XVIIe siècles). *Rev Med Picardie* 19(1):9–204.
48. De La Lande J (1762) *Art de faire le parchemin par M. de la Lande* (De l'imprimerie de H. L. Guerin et L. F. Delatour).
49. Hockey SF (1975) *The account-book of Beaulieu Abbey* (CAMDEN FOURTH SERIES, LONDON OFFICES OF THE ROYAL HISTORICAL SOCIETY).
50. Vallat F (2009) Les Bøeufs malades de la peste, la peste bovine en France et en Europe, XVIIIe-XIXe siècle. Available at: https://hal.archives-ouvertes.fr/hal-00523795/.
51. Binois A (2015) Excavating the history of ancient veterinary practices. *Vet Rec* 176(22):564–569.
52. Strohalm M, Kavan D, Novák P, Volný M, Havlícek V (2010) mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data. *Anal Chem* 82(11):4648–4651.
53. Buckley M, Collins M, Thomas-Oates J, Wilson JC (2009) Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Rapid Commun Mass Spectrom* 23(23):3843–3854.
54. Buckley M, et al. (2010) Distinguishing between archaeological sheep and goat bones using a single collagen peptide. *J Archaeol Sci* 37(1):13–20.
55. Fischer R, Kessler BM (2015) Gel-aided sample preparation (GASP)--a simplified method for gel-assisted proteomic sample generation from protein extracts and intact cells. *Proteomics* 15(7):1224–1229.

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