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Rapid Communications in Mass Spectrometry

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Using MALDI-FTICR Mass Spectrometry to Enhance ZooMS Identifications of Pleistocene Bone Fragments Showing Variable Collagen Preservation

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Keywords: burnt bones | collagen preservation | MALDI-CASI-FTICR (Continuous Accumulation of Selected Ions) | Pleistocene | PRM (Parallel Reaction Monitoring) | ZooMS

ABSTRACT

Rationale: Recent advances in high-throughput molecular analyses of collagen peptides, especially ZooMS (Zooarchaeology by Mass Spectrometry), have permitted breakthroughs in the analysis of archaeological material that is highly fragmented, a factor that hinders morphological identification. Despite these advances, the challenge of successfully analysing archaeological samples with poorer collagen preservation persists. This paper examines the potential of two mass analysers, TOF (*Time of Flight*) and FTICR (*Fourier-transform ion cyclotron resonance*), and addresses how they can be used to optimise the ZooMS workflow.

Methods: Type 1 collagen (COL1) was extracted from 89 archaeological bones from the French Palaeolithic site of Le Piage (37–34 ka cal BP). Three ZooMS extraction protocols were applied, an acid-free buffer method (AmBic), offering rapid and less destructive analysis, and two methods of acid demineralisation (HCl and TFA) that provide higher peptide resolution. After analysing the specimens with MALDI-TOF and MALDI-FTICR, we used bottom-up and PRM (*Parallel Reaction Monitoring*) LC–MS/MS, and MALDI-CASI-FTICR (*Continuous Accumulation of Selected Ions*) to verify 26 ambiguous identifications.

Results: Overall, 99% of the samples could be identified to at least family level, with the rate of identification and precision varying by method. Despite challenges in detecting specific biomarkers with MALDI-FTICR—especially peptide A (COL1α2 978–990), which tends to be unstable and poorly ionised—the high resolution of this method allowed the successful identification of more degraded specimens, including burnt bones.

Conclusions: Our work highlights the robustness of traditional MALDI-TOF ZooMS for retrieving collagen and for providing taxonomic identifications with low failure rates, features that are critical when processing large numbers of samples. MALDI-FTICR shows better potential when working with precious samples or degraded collagen. This study advances the analytical

detection of peptides by optimising the ZooMS workflow and by tailoring it to specific archaeological contexts showing variation in degree of preservation.

1 | Introduction

Morphologically unidentifiable faunal remains are commonly found in archaeological sites. Their high degree of fragmentation in Palaeolithic contexts poses challenges for the interpretation of site formation processes and human activity. Recently, the use of biomolecular analyses, such as palaeoproteomics [1], has proven effective in overcoming these challenges [2]. Proteins are abundant in ancient skeletal tissues and can preserve for millions of years [3]. This potential for preservation has sparked a surge of research and the establishment of the field of palaeoproteomics [4–9]. Type I collagen (COL1) is the most abundant protein in skeletal material and is highly stable [1, 9]. For this reason, a range of palaeoproteomic methods, notably ZooMS (Zooarchaeology by Mass Spectrometry), have been developed to identify archaeological faunal remains using variations in collagen peptide composition [10–12]. ZooMS is a collagen-based, high-throughput method, which relies on Matrix-assisted laser desorption ionisation (MALDI)-Time of Flight (TOF) mass spectrometry and peptide mass fingerprinting (PMF).

Since the emergence of ZooMS, various lab protocols have been developed in response to a range of methodological issues, including the evaluation of the impact of sampling techniques, extraction and digestion methods on peptide detection [13–22]. Few studies have compared mass spectrometry techniques despite their importance in elaborating novel applications [10, 23, 24]. In the ZooMS approach, the MALDI soft ionisation source is preferred because it performs better at capturing high molecular masses contained in collagen [22–24]. and its low costs allow for the rapid analysis of very large sets of samples (hundreds to thousands of specimens). Here, we compare two analysers coupled with a MALDI source, the MALDI-TOF (TOFMS) and the high-resolution MALDI-FTICR (Fourier-transform Ion Cyclotron Resonance, FTICRMS), and examine how they perform when using three different extraction procedures (Figure 1). Rather than providing a comprehensive comparison of all the various protocols currently in use, the aim of our study is to assess how the MALDI-FTICR mass spectrometer can enhance traditional ZooMS methodologies, and whether it can be used to develop a high-resolution workflow tailored specifically at degraded archaeological samples. We also used LC–MS/MS (using Data Dependant Analysis [DDA] and Parallel Reaction Monitoring [PRM]) and MALDI-CASI-FTICR (Continuous Accumulation of Selected Ions) mass spectrometry to verify taxonomic identifications that were ambiguous (Figure 1).

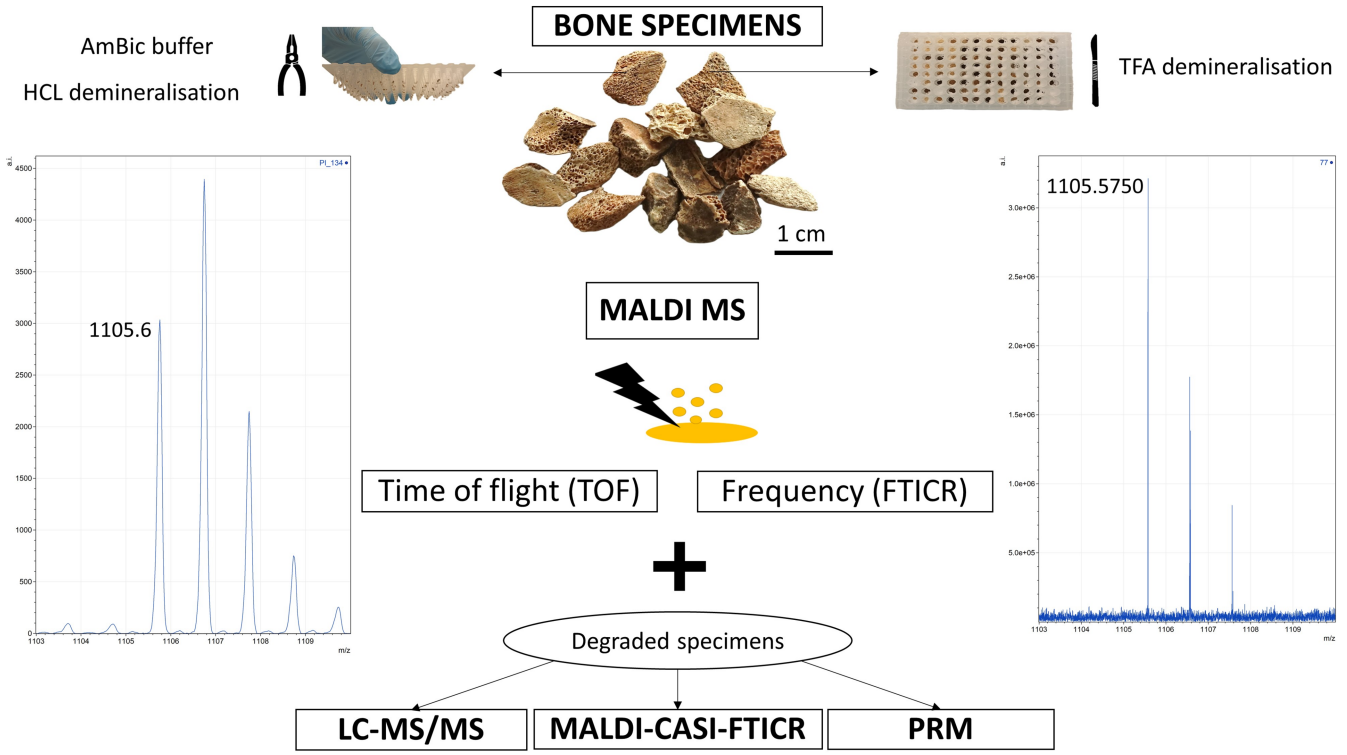

FIGURE 1 | Simplified workflow for the collagen extraction procedures and mass spectrometry techniques (MALDI-TOF and MALDI-FTICR) applied to a sample of indeterminate bone fragments from Le Piage, France. This figure illustrates the three collagen extraction protocols (AmBic, TFA and HCl) and the two mass spectrometry approaches (MALDI-TOF and MALDI-FTICR) used in the analysis. The lower left and right panels show Peptide Mass Fingerprinting (PMF) of peptide P1 (α1 508, *m/z* 1105) for a single sample (PI-134-ICR) analysed with both mass spectrometers. The isotopic distributions are markedly different in these panels due to the separation by MALDI-FTICR of the two components of the first isotope. The spectrum shows a ^{13}C and deamidated peptide peaks separated by 0.019 *m/z*. [Colour figure can be viewed at wileyonlinelibrary.com]

We applied these methodological approaches to a sample of Early Aurignacian (37–34 ka cal. BP) bone fragments recovered during recent excavations at the site of Le Piage (Lot, France) [25–28]. Previous collagen-based analyses at the site, including radiocarbon dating and ZooMS analyses [29, 30], revealed relatively poor collagen preservation, a pattern possibly explained by taphonomic processes (e.g., differing levels of sub-aerial weathering related to changes in moisture or temperature). Therefore, this material provides an ideal, well-contextualised case study to address challenges posed by collagen preservation, a common issue in Palaeolithic contexts [31–33].

2 | Material and Methods

2.1 | Site Background

Le Piage (Lot, France) is an open-air site located in southern France with a well-preserved archaeological sequence [26]. Despite initial reports arguing for the presence of inter-stratifications of distinct techno-complexes [25, 28], recent excavations demonstrate a clear succession of Proto-Aurignacian (42–39 ka BP), Early Aurignacian (37–34 ka cal. BP) and Solutrean-Badegoulian (22.0–24.5 ka cal. BP) occupations [26]. During the Early Aurignacian, the site appears to have been used primarily for short-term visits by small groups with hunting activities mostly focused on reindeer exploitation, the dominant species in the faunal assemblage [29]. Despite its position within a limestone karstic system, open-air exposure as well as cyclic water inclusions from the La Relinquière stream appear to have negatively affected the preservation of collagen [30]. This was illustrated both through failed radiocarbon dates, and variable identification rates and glutamine deamidation values in a previous ZooMS study [31].

2.2 | Sample Selection

This study analyses 89 morphologically unidentifiable bone fragments stored at the University of Bordeaux, which were first analysed as part of a larger ZooMS study aimed at investigating species composition [31]. All fragments from this larger study are 1–2 cm in maximum length and were selected at random from the material recovered through water screening from a single 25×25-cm sub-square of layer GI (Early Aurignacian). For the methodological study presented here, a subsample was selected to include different taxa and different degrees of collagen preservation (based on the previous ZooMS identifications) [31]. This also includes five fragments showing black surface colouration likely induced by half or full carbonisation, which we assigned to Stiner's stage 2–4 [34]. Because of this burnt appearance and the fact that heat negatively affects collagen preservation, these fragments were only analysed with the acid-based protocols (HCl and TFA) using MALDI-MS and LC–MS/MS to optimise collagen extraction.

2.3 | Chemicals, Preparation of Compounds and Buffer Solutions

The ammonium bicarbonate (AmBic) buffer was purchased from Sigma-Aldrich (Saint-Louis, MO, USA) for a 50-mM buffer with a pH of 8.8. Trifluoroacetic acid (TFA, Carl Roth, Karlsruhe, Germany) and hydrochloric acid (HCl, Sigma-Aldrich) were used for demineralisation solutions (% (v/v) TFA and 0.6 M HCl). The bovine trypsin was purchased from Promega (Madison, WI, USA), while the HypersSep C18 96 well plates and AttractSPE®Disks 96 wells C18 are from Thermo Fischer Scientific (Waltham, MA, USA) and Affinisep (Le Houlme, France), respectively. The matrix a-cyano-4-hydroxycinnamic acid (HCCA) was obtained from VWR (Radnor, PA, USA). All water samples were of Ultra High Quality (UHQ) and were obtained from Milli-Q® IQ 7003/7005 Water Purification Systems (Merck Millipore, Burlington, Massachusetts, United States) or derived by water filtration with a two-stage Millipore system (Milli-Q® Academic with Q-Gard 1 and Progard 2 cartridges, Merck Millipore, Burlington, Massachusetts, United States) at the University of Lille.

2.4 | Experimental Setting

All sampling, collagen extraction and peptide purification were conducted in the Palaeoproteomics laboratory of the Chaire de Paléoanthropologie, Collège de France, Paris, France. The TFA protocol was performed in the Miniaturization for Synthesis, Analysis and Proteomics unit (MSAP), University of Lille, France. All specimens were sampled twice to test three different protocols (Table SI 1). Bone chips of approximately 5–10 mg were obtained using pliers and put in 96-well plates [35]. The same bone chips were analysed successively applying the AmBic [22] and HCl demineralisation [11] protocols. The original bone was subsequently scraped with a scalpel to sample ~5 mg of bone powder transferred into a MSIPS4510 plate (Merck Millipore, Burlington, MA, USA) immersed in TFA solution for demineralisation [10]. The last well of each plate was left empty as a control for contamination.

2.4.1 | AmBic Protocol

The AmBic protocol is based on Van Doorn et al. (Table S1) [22]. In each sample, 100 µL 50 mM AmBic (pH: 8.8) was added, left overnight and discarded. Then, the samples were incubated in 100 µL of AmBic at 65°C for 1 h. The plate was centrifuged and 50 µL of supernatant was collected to add 1 µL of trypsin (Promega) at 37°C for 16 to 18 h. The bone sample was subsequently stored in the remaining 50 µL of AmBic at −20°C. To halt digestion, 1 µL of 10% trifluoroacetic acid (TFA) was added. The samples were cleaned in HyperSep C18 96-well plates using a HyperSep™ Universal Vacuum Manifold. The filtered peptides were finally eluted in 100 µL of conditioning solution (0.1% TFA in 50:50 ACN/water v/v).

2.4.2 | HCL Protocol

The remaining bone chips, collected in 50 µL of AmBic, were demineralised in 120 µL of 0.6 M HCl acid overnight and rinsed three times with 100 µL 50 mM AmBic (pH: 8.8) following the protocol described in Buckley et al. [11] The subsequent steps replicate those for the AmBic extraction protocol (Table S1).

2.4.3 | TFA Protocol

The protocol of demineralisation with TFA is described in Bray et al. [10]. Briefly, 0.45-μm MultiScreenHTS-IP 96 wells plates (MSIPS4510, Millipore, Billerica, MA, USA) was hydrated with 70% ethanol. All solutions were eluted through the wells using a vacuum manifold (Merck KGaA, Darmstadt, Germany) pumped by a DS 102 rotary vane pump (Agilent, Santa Clara, CA, USA). Then, <5 mg of bones were deposited and demineralised with 100 μL of TFA 5% at 4°C and left overnight. The demineralisation solution was recovered in 96-well plates. The bones were subsequently washed with 100 μL of AmBic 50 mM (pH: 8.8) and gelatinised at 65°C during 1 h. After this, 8 μL of NaOH 6 M were added followed by 100 μL of AmBic 100 mM (pH: 8.8) added to the demineralisation solution. The bones and the demineralised solution were digested with trypsin at 37°C left overnight. The peptides from bones and the demineralised solution were purified on C18 96-well plates (Affinisep). The concentration was then estimated by measuring the OD at 215 nm using 1 μL of the solution with the aid of a droplet UV spectrometer (DS-11+, Denovix, Wilmington, DE, USA). In this paper, the results of this protocol are only presented for the bone powder as the analysis of the demineralisation solution did not provide additional peaks useful for PMF.

2.5 | Mass Spectrometry Instruments

A MALDI-TOF 5200 AB Sciex (Framingham, MA, USA) in the High School of Physics and Industrial Chemistry (ESPCI) in Paris, France, and a Bruker 9.4 Tesla SolariX XR FTICR mass spectrometer controlled by ftmsControl software (equipped with a CombiSource and a ParaCell, Bruker Daltonics, Bremen, Germany) in the MSAP, University of Lille, France, were used for the analyses. For the additional LC–MS/MS analyses, we used an Orbitrap Q Exactive plus Mass Spectrometer hyphenated to a U3000 RSLC Nanofluidic HPLC System (ThermoFisher Scientific, Bremen, Germany) based at the MSAP laboratory at the University of Lille, France.

2.5.1 | TOFMS

MALDI ionisation allows the detection of intact peptides, and for this reason, has widely been used in ZooMS studies. This apparatus is usually coupled with a TOF analyser [11, 20, 34–37] in order to cover the spectrum of high mass molecules contained in digested collagen [23, 38, 39]. We deposited 0.5 μL of peptide solution on 384-well AB Sciex MALDI plates in triplicates, except for the TFA extraction deposited only once for comparison with the MALDI-FTICR. Next, we mixed the solution with 0.5 μL of HCCA matrix (10 mg/mL; 0.1% TFA in 50:50 ACN/water v/v) and let the spotted solutions dry in a fume hood. The calibrant (Proteomix Peptide calibration mix4, LaserBioLabs, Sophia Antipolis, France) was mixed with the same matrix (1 mg/2 mL) and spotted on the 13 dedicated spots disseminated on the MALDI plate. The external plate model calibration was performed on four peptides bradykinin fragment 1–5, 573.315 Da: human angiotensin II (1046.542 Da), neurotensin (1672.917 Da), ACTH fragment 18–39 (2464.199) and oxidised insulin B chain (3494.651 Da) for each of the 13 spots. The calibration was performed manually and checked automatically within a precision of 50 ppm. For MALDI MS sample measurements, laser intensity was set at 50% after optimisation of signal-to-noise ratio on several spots, then operated at up to 3.000 shots accumulated per spot, and covering a mass-to-charge range of 800 to 3500 Da. The analysis was run in an automatic mode after a manual control of 10 samples. The triplicate spectra obtained from the MALDI-TOF were merged [18], smoothed and converted in RStudio using the MALDIquant and MALDIquantForeign packages [40]. The software mMass (V.5.5.0) was used to analyse the spectra (http://www.mmass.org/) [41] with a signal-to-noise (S/N) ratio set at 3.0 to enhance peak detection [42].

2.5.2 | FTICRMS

The FTICR analyser is a high-resolution ion cyclotron resonance analyser [43]. It is commonly used for the analysis of complex samples, such as petroleum, in instances where high resolution is required to differentiate compounds that number in the thousands [43]. More recently, it has been integrated in cultural heritage studies [44, 45], although its application to archaeological specimens remains limited [10]. FTICR analysis is based on ion cyclotron frequency, which is converted in a mass spectrum [43]. The FTICR analyser measures peptide masses with greater accuracy and at a higher resolution than a TOF analyser. For these reasons, it typically provides more precise and more secure ZooMS identifications [10]. To perform FTICR analyses, we deposited 1 μL of peptide solution on 384 Ground steel MALDI plates (Bruker Daltonics) on which 1 μL of HCCA matrix (10 mg/mL in ACN/H_2O 80:20 v/v 0.1% TFA) was added. The spots were then left to dry at room temperature. To reduce costs, triplicates were not run as this is not standard for MALDI-FTICR. The MALDI-FTICR analyser was equipped with a Bruker Smartbeam-II™ laser system that operated at a frequency of 1000 Hz. The predefined shot pattern for irradiation was set at 'medium.' Each mass spectrum was obtained from the automatic merging of 10 scans of 300 laser shots using 2 M data points and a mass range of m/z 700 to 4500 (FID (Free Induction Decay) = 5.128 s). The laser power was set to 32%. The MALDI-FTICR was calibrated with a saturated solution of red phosphorus in ACN. The software Data Analysis Bruker 5.0 was used to read the MALDI-FTICR spectra with S/N set at 3.0, and quality at 0.6.

2.5.3 | Bottom-Up LC–MS/MS

The LC–MS/MS methods were performed as described in Bray et al. [46] on a subsample of 26 specimens for which the MALDI-MS analysis resulted in ambiguous identifications. To perform this analysis, we used the HCl collagen extracts stored in the freezer after the MALDI-MS analyses to avoid the invasiveness of a third round of sampling. Samples were diluted at a concentration of 1 μg/μL before LC–MS/MS analysis. LC–MS/MS analyses were performed on an Orbitrap Q Exactive plus mass spectrometer hyphenated to a U3000 RSLC Microfluidic HPLC System (ThermoFisher Scientific, Waltham, Massachusetts, USA). One microlitre of the peptide mixture at a concentration of 1 μg/μL was injected with solvent A (5% acetonitrile and 0.1% formic

acid v/v) for 3 min at a flow rate of 10 μL·min^{-1} on an Acclaim PepMap100 C18 pre-column (5 μm, 300 μm i.d. × 5 mm) from ThermoFisher Scientific. The peptides were then separated on a C18 Acclaim PepMap100 C18 reversed phase column (3 μm, 75 μm i.d. × 500 mm) using a linear gradient (5%–40%) of solution B (75% acetonitrile and 0.1% formic acid) at a rate of 250 nL·min^{-1}. The column was washed with 100% of solution B during 5 min and then re-equilibrated with buffer A. The column and the pre-column were placed in an oven at a temperature of 45°C. The entire analysis was completed in approximately 140 min. The LC runs were performed in positive ion mode with MS scans from *m/z* 350 to 1500 in the Orbitrap mass analyser (resolution: 70000, *m/z* 200). The automatic gain control was set at 1e10^6. MS/MS scans were sequentially acquired in a high-energy collision dissociation cell for the 15 best detected ions in the full MS survey scan. Automatic gain control was set at 5e10^5, and the normalised collision energy was set to 28 eV. Dynamic exclusion was set at 90s. Ions with more than eight charges were excluded.

Bioinformatics analysis of the raw LC–MS/MS forms was performed with Proteome Discoverer™ 2.4 (ThermoFisher Scientific) using the Sequest HT search engine. The sequences were first interrogated against the Swissprot database, which contains 571,282 entries (release 2024_02). Then, the sequences were compared with the COL1A1 and COL1A2 markers for *Bos taurus* reported in the Uniprot database (accession number P02453, P02465 respectively), while for *R. tarandus*, we used the sequences from Genome assembly RanTarSib_v1_BIUU (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_004026565.1/) (Data S1, sequences Rangifer_Bos) because the published Swissprot sequence is incomplete [12]. The precursor's mass tolerance was set at 10 ppm, and fragment ion mass tolerance, to 0.02 Da. Semi-trypsin mode was set to an enzyme. Variable modifications were set for hydroxyproline (P, +15.995 Da), oxidation of methionine (M, +15.995 Da), deamidation of glutamine and asparagine (N, Q, +0.98 Da).

2.5.4 | Targeted Peptides: LC–MS/MS PRM and MALDI CASI FTICR MS

The 26 samples submitted to DDA LC–MS/MS were also analysed using PRM (Parallel Reaction Monitoring) LC–MS/MS and MALDI-CASI-FTICR to target specific biomarkers. The PRM LC–MS/MS analyses [47] were performed with the same concentration of peptides, injection volume and mass spectrometer as for bottom-up LC–MS/MS. However, the total duration of the analysis was limited to 70 min. We used a PRM workflow with one MS1 full scan (*m/z* 350–1500) and scheduled MS/MS fragmentation of six ZooMS markers (Data S2, data for PRM and CASI). The MS spectra were acquired with AGC at 1e10^6, ion time injection was 90 ms set at 70000 resolution. To obtain MS/MS spectra, AGC was set at 5e10^5, ion time injection was 200 ms set at 17500 resolution. Isolation windows were completed at *m/z* 2 and the energy for HCD fragmentation was performed at 30 eV. LC–MS/MS data were processed by Quant Browser (Thermo) and Skyline [48]. Peak intensities were used for peptide identification. The four best resolved fragment ions were used for sequence identification. The full peptide sequences used for the PRM and MS/MS spectra of the six ZooMS markers that we selected for PRM are provided in the supplementary dataset (Data S2, data for PRM and CASI).

Each mass spectrum from MALDI-CASI-FTICR [48, 49] analysis was obtained from 20 scans of 500 laser shots using 1 M data points and a mass range of *m/z* 700 to 3500 (FID = 2.564 s). Six ions were selected: *m/z* 1105.6, 1150.6, 1166.6, 1192.7, 1208.7 and 1427.7, with isolation windows at *m/z* 10. The full peptide sequences used for CASI are given in the supplementary dataset (Data S2, data for PRM and CASI).

2.6 | PMF for Le Piage Taxa

The taxonomic identifications were made manually by PMF, based on a published database [12] which records the masses of nine peptide markers for mammalian species. In the analysis, we followed the recent nomenclature to name these peptide markers (GVQGPPGPAGPR, COL1α1 508–519 [P1]; GLTGPIGPPGPAGAPGDKGE(**A/T**)GPSGPAGPTGAR, COL1α1 586–618 [F]; GSTGEIGPAGPPGPPGLR, COL1α2 292–309 [P2]; GEQGPAGPPGFQGLPGPAGTAGEAGKPGER, COL1α2 454–483 [E]; GIPGEFGLPGPAGAR, COL1α2 484–498 [B]; GPPGESGAAGPTGPIGSR, COL1α2 502–519 [C]; GPSGEPGTAGPPGTPGPQG(**L/F**)LG(**A/P**)PGFLGLPGSR, COL1α2 757–789 [G]; GLPGVAGSVGEPGPLGIAGPPGAR, COL1α2 793–816 [D]; (**I/A**)GQPGAVGPAGIR, COL1α2 978–990 [A]) [50]. The peptides have masses between *m/z* 800 to 3500, which corresponds to approximately 8 to 30 amino acids per peptide [35]. Because AmBic tends to produce peaks of lower intensity with MALDI-TOF—a problem that impedes spectral analysis—they were not analysed on MALDI-FTICR. As our aim was to explore potential future ZooMS applications rather than extensively test all possible protocols with these mass spectrometers, we prioritised testing HCl with MALDI-FTICR to enhance ZooMS identifications on archaeological material that derive from challenging contexts.

In a PMF-based analysis, all biomarkers are potentially crucial, as their specific associations provide the basis for taxonomic identification. In this study, we recorded the presence of nine markers for each specimen and protocol (Data S3, recording of the peptide markers). Some peptides, such as peptide A (COL1α2 978–990), were essential for distinguishing *Bos/Bison* from reindeer, the most common taxon at the site (Figure 2) [12, 29, 31]. Peptide A (COL1α2 978–990) is identified by a pair of markers (*m/z* 1150.6 and 1166.6 for reindeer; *m/z* 1192.7 and 1208.7 for *Bos/Bison*) [12] that are the products of hydroxylation of a proline, which causes a 15.99 mass shift of the marker. However, peptide A (COL1α2 978–990) can be difficult to identify, as one or both peaks may be poorly ionised. High-mass peptide markers (*Bos/Bison*: peptide F [COL1α1 586–618], *m/z* 2853–2869; peptide G [COL1α2 757–789], *m/z* 3017–3033; Reindeer: peptide F [COL1α1 586–618], *m/z* 2883–2899; peptide G [COL1α2 757–789], *m/z* 3077–3093) are also critical for the identification of *Bos/Bison* and reindeer, but their presence is also constrained by collagen preservation. When these peptide markers were poorly ionised, the specimens were assigned to *Bovidae*/reindeer (in the presence of peptide C [COL1α2 502–519]) or Bovidae/Cervidae (in the absence of peptide C [COL1α2 502–519]) [28], (Table S2). Equidae are also present at Le Piage; these can be distinguished from other taxa by using peptide marker D (COL1α2 793–816, *m/z* 2145.1) [12, 31].

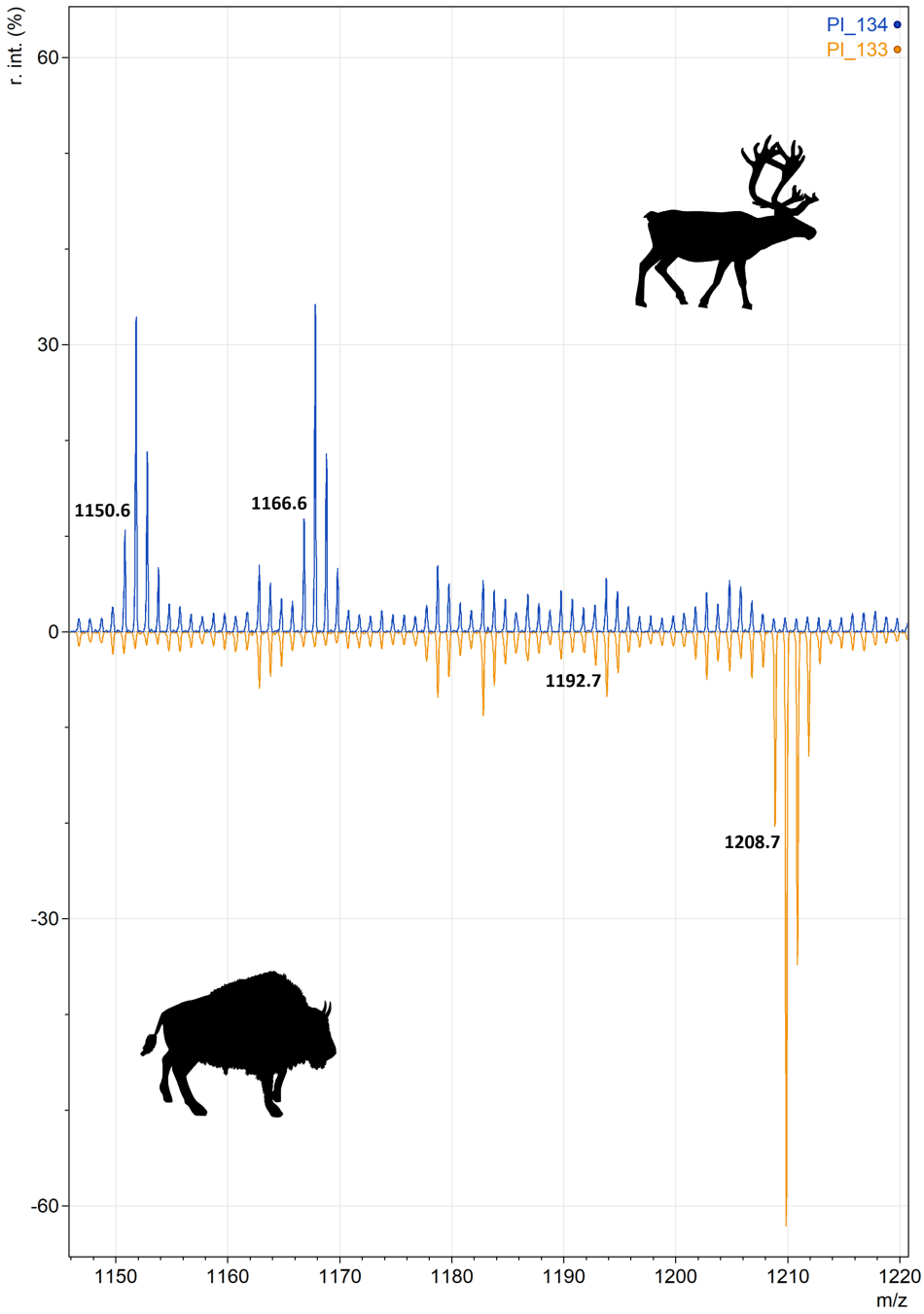

FIGURE 2 | Examples of peptide A (COL1α2 978–990) *m/z* for two specimens analysed with HCl-MALDI-TOF MS. At the top, in blue, specimen PI-134 with peptide A shows 1150.6 and 1166.6 *m/z* peaks that are characteristic of reindeer. At the bottom, in orange, specimen PI-133 with peptide A shows 1192.7 and 1208.7 *m/z* peaks that are specific to *Bos/Bison*. [Colour figure can be viewed at wileyonlinelibrary.com]

3 | Results

3.1 | Differential Identification Rates

The three protocols (AmBic, HCl and TFA) coupled with two MALDI mass spectrometry techniques (TOFMS and FTICRMS) produced ZooMS identification rates ranging from 71.4 to 91.7% (Table 1). Combining all approaches, 99% of the bones were identified to at least family level (see full list of identifications in Data S4). The rate of identification varied by type of protocol and mass spectrometer used, with certain approaches outperforming others in their ability to separate Bovidae from reindeer or Cervidae. Specimens assigned to Bovidae/reindeer and Bovidae/Cervidae were included in the sample of identified specimens, as they can yield insights on the taxonomic nature of the specimens and can be used to exclude other attributions. Interestingly, both the lowest (71.4%, HCl protocol) and highest (91.7%, TFA protocol) rates of identifications were observed with the MALDI-FTICR analyser (Table 1), which suggests that the efficacy of a mass spectrometer also depends on the extraction protocol.

TABLE 1 | Rates of ZooMS identifications in the study sample ($n = 89$ bone fragments). Note that the sample for the AmBic protocol excludes five burnt bone fragments ($n = 84$ bone fragments).

ZooMS ID	AmBic + TOF	%	TFA + TOF	%	HCl + TOF	%	TFA + FTICR	%	HCl + FTICR	%
Unburnt bones ($n = 84$)										
Reindeer	54	*64.3%*	40	*47.6%*	48	*57.1%*	36	*42.9%*	33	*39.3%*
Bos/Bison	5	*6.0%*	6	*7.1%*	5	*6.0%*	6	*7.1%*	3	*3.6%*
Equidae	1	*1.2%*	1	*1.2%*	1	*1.2%*	2	*2.4%*		
Bovidae/Reindeer	1	*1.2%*	16	*19.0%*	10	*11.9%*	31	*36.9%*	18	*21.4%*
Bovidae/Cervidae	3	*3.6%*					2	*2.4%*	6	*7.1%*
Total Identified	64	*76.2%*	63	*75.0%*	64	*76.2%*	77	*91.7%*	60	*71.4%*
Total non-identified	20	*23.8%*	21	*25.0%*	20	*23.8%*	7	*8.3%*	24	*28.6%*
Burnt bones ($n = 5$)										
Reindeer										
Bos/Bison							2			
Equidae										
Bovidae/Reindeer							2			
Bovidae/Cervidae							1		2	*40%*
Total identified	x		0	*0%*	0	*0%*	5	*100%*	2	*40%*
Total non-Identified	x		5	*100%*	5	*100%*	0	*0%*	3	*60%*

Note: Italics are used for species/genus taxonomic terminology. It is also used to differentiate percentages and counting numbers.

3.1.1 | Identifications on the MALDI-TOF Analyser

To replicate the standard procedure used in ZooMS, we compared the MALDI-TOF taxonomic identifications obtained with three different protocols (AmBic, HCl and TFA). We began with the less destructive AmBic protocol, an approach in general complemented by the HCl protocol when additional or improved identifications are needed. We also considered the TFA protocol—commonly used with MALDI-FTICR [10]—to evaluate its compatibility with MALDI-TOF. Our results show similar rates of identifications (>75%) for the three methods, although the taxonomic distributions are significantly different according to a chi-square test of independence (Table 1; AmBic+TOF vs. TFA + TOF, $\chi^2 = 18.4$, $p < 0.01$; AmBic+TOF vs. HCL + TOF, $\chi^2 = 10.7$, $p < 0.05$). The adjusted standardised residuals indicate that reindeer is significantly more common in the AmBic data (AmBic + TOF vs. TFA + TOF = +2.68, $p < 0.01$; AmBic + TOF vs. HCL + TOF = +1.32, $p = 0.0937$), whereas 'Bovidae/reindeer' are more abundant in the TFA and HCL data (AmBic + TOF vs. TFA + TOF = +3.94, $p < 0.001$; AmBic + TOF vs. HCL + TOF = +2.84, $p < 0.01$).

3.1.2 | Identifications on the MALDI-FTICR Analyser

When applying TFA and HCl demineralisation protocols with MALDI-FTICR, the rate of identification for the HCl-MALDI-FTICR protocol (71.4%, Table 1) is lower than that obtained with TFA-MALDI-FTICR (91.7%, Table 1); however, the difference is not statistically significant (HCl + FTICR vs. TFA + FTICR, $\chi^2 = 6.6$, $p > 0.05$). The higher rate of identification obtained with TFA-MALDI-FTICR is slightly deceptive given the high representation (36.9%) of 'Bovidae/reindeer'—a poorly resolved taxon—in the sample. This caveat aside, we note that TFA-MALDI-FTICR produced fewer spectra lacking any collagen markers (Table 1), including a single case of a blank spectrum, relative to the HCl protocol (Data S3, recording of the peptide markers).

3.2 | Peptide Markers Presence and Intensity

In open-air contexts like Le Piage, the identification of peptide markers tends to be challenging [36]. When assessing the presence/absence of the nine main ZooMS markers across our samples (Figure 3 and Data S5, peptide markers), we can make three main observations (Figure 3):

i. When collagen was recovered, the HCl-MALDI-TOF yielded the most robust and precise identifications and was best at detecting all nine diagnostic peptide markers.

ii. Failures (spectra with two or fewer diagnostic peaks) were less common with the TFA-MALDI-FTICR approach, which indicates improved collagen extraction. However, the proportion of imprecise identifications (e.g., Bovidae/reindeer) was relatively high with this approach.

iii. With an average of seven peptide biomarkers per spectrum, the less invasive AmBic-MALDI-TOF approach generated precise identifications. However, the number of detected peptide markers is highly variable (ranging from 0 to 9) as this method is probably most affected by collagen preservation issues.

With both mass spectrometers, the use of the HCl protocol increased homogeneity in peak detection at both low (< *m/z*

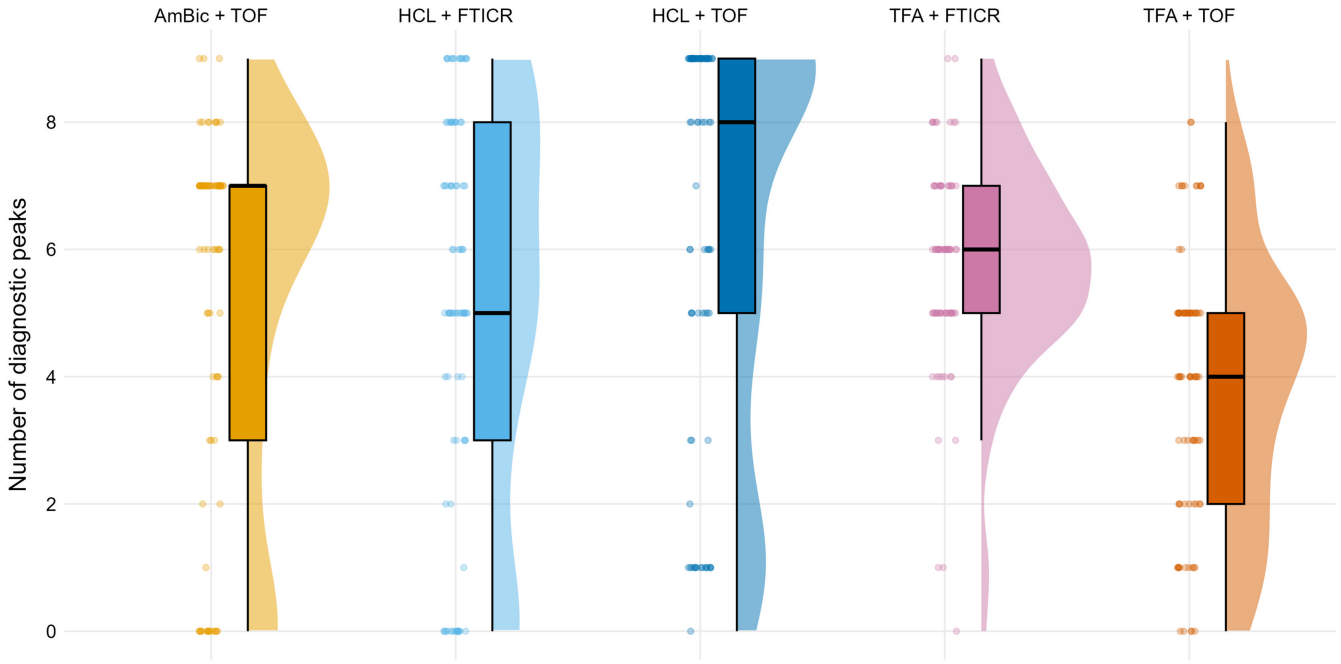

FIGURE 3 | Boxplots illustrating the total number of diagnostic peptide markers (maximum 9) detected per protocol ($n = 84$ specimens, burnt bones excluded). Each specimen is identified by a dot. The boxplots indicate the median and quartiles, while the violins show the distribution of the specimens around the median. Taxonomic identification becomes more reliable as the number of detected peaks increases. Failed spectra are ones with two or fewer diagnostic peptide markers. [Colour figure can be viewed at wileyonlinelibrary.com]

2500, TOFMS: 74%; FTICRMS: 84%) and high (> *m/z* 2500, TOFMS: 55%; FTICRMS: 30%) masses (Figures 4 and S5). The AmBic protocol coupled with MALDI-TOF was efficient at detecting low (72%, Data S5), but not high (28%, Data S5) mass peptides. This problem was amplified with the TFA-MALDI-TOF method, as it yielded an even lower rate of detection of high mass peptides (5%, Data S5), possibly because the collagen solutions were deposited only once—not in triplicates—to make the protocol comparable to that of the MALDI-FTICR data obtained using TFA solutions. The TFA-MALDI-FTICR protocol was most efficient at detecting peptides with masses between *m/z* 1400 and *m/z* 2300 (Figure 4). Regardless of the method, we note that when the spectra are most challenging, possibly due to poorer collagen preservation, the peptides with the highest masses are typically those that were missing from the spectra [19], a trend best exemplified by the MALDI-FTICR data. Our results also seem to confirm that MALDI-TOF outperforms the other methods when the peptidic solutions are spotted in triplicates. Overall, peptide A (COL1α2 978–990)—a crucial marker for differentiating *Bos/Bison* from reindeer—was best detected in our study using MALDI-TOF with the AmBic protocol (Figure 4).

Given the pivotal role played by peptide A (COL1α2 978–990) in our analysis, we tried to accurately quantify its presence. MALDI-FTICR spectra are ideal for that, as they yield the exact mass of the peptide (*m/z* 1166.6742 or *m/z* 1208.6746). To provide an objective measure, we developed an intensity ratio to quantify the minimum intensity required for the detection of peptide A (COL1α2 978–990) relative to peptide P1 (COL1α1 508–519), a marker of collagen presence in mammals (Data S6, relative intensity of ratio peptide A/P1). However, the measure was inconclusive, likely because preservation and peak expression were too variable between samples.

3.3 | Control and Precision of the MALDI MS Results Using DDA and PRM LC–MS/MS and MALDI-CASI-FTICR

Complementary MS analyses were conducted on a subset of degraded samples ($n = 26$, including five burnt fragments) that could only be assigned to 'Bovidae/reindeer' using TFA-MALDI-FTICR. These specimens were reanalysed here with the goals of (i) confirming the presence of endogenous collagen, (ii) improving the analytical distinction between reindeer and *Bos/Bison* and (iii) assessing the confidence limits for the detection of MALDI-FTICR peaks.

3.3.1 | DDA LC–MS/MS

Of the 26 samples analysed using LC–MS/MS, 13 resulted in positive identifications (see list of identifications in Data S4). Of these, 12 were assigned to reindeer, and one, to *Bos* sp. Except for samples PI-175-ICR and PI-186-ICR, both attributed to Bovidae/reindeer, the other specimens had all previously been identified as deriving from reindeer or *Bos/Bison* with one or more protocols. Failures with LC–MS/MS mostly involved highly degraded specimens. The specimen identified as *Bos* sp. with LC–MS/MS had consistently been assigned to *Bos/Bison* regardless of the MALDI-TOF protocol (AmBic, TFA, and HCl), which supports the new attribution. In contrast, specimens identified as Bovidae/reindeer using

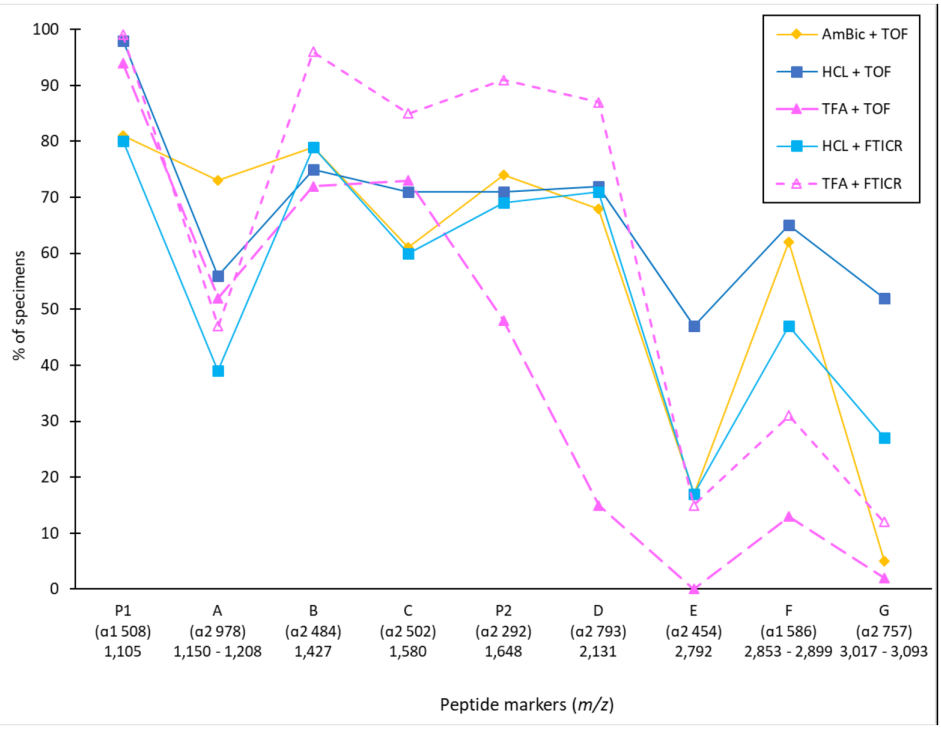

FIGURE 4 | The nine peptide markers used for identifying mammalian species in this study and their occurrence (%) in the study assemblage as a function of extraction protocol (AmBic, HCl or TFA) and MALDI mass spectrometer (TOFMS or FTICRMS). [Colour figure can be viewed at wileyonlinelibrary.com]

the TFA-MALDI-FTICR method—given the dual presence of *m/z* 1166.6276 and *m/z* 1208.6746 peaks—were all assigned to reindeer. This result confirms the difficulty of identifying peptide A (COL1α2 978–990) in degraded samples.

3.3.2 | PRM LC–MS/MS and MALDI-CASI-FTICR MS

To better target the presence of peptide A (COL1α2 978–990) and refine the taxonomic identifications of those degraded samples, PRM LC–MS/MS and MALDI-CASI-FTICR were performed (Figures S1–S8: MS/MS spectra for targeted ions with PRM LC–MS/MS). The identifications obtained with these approaches are internally consistent (Table S3). Relative to the preceding methods, MALDI-CASI-FTICR and PRM LC–MS/MS allowed the identification of one and two additional specimens (Data S4, list of all identifications), respectively. These taxonomic attributions were based on the presence of peptides P1 (COL1α1 508–519) and A (COL1α2 978–990). As these more sensitive methods target peptides, they provide an independent approach to the MALDI-FTICR and DDA LC–MS/MS methods. Our study, which represents the first application of MALDI-CASI-FTICR-MS to Palaeolithic remains, suggests that the approach has considerable potential for improving taxonomic identification in contexts of variable or poor collagen preservation.

3.3.3 | Burnt Bones

Collagen in bone tends to break down when heated, leading to significant loss of organic matter, particularly when bones are heavily carbonised or calcined [51–53]. For this reason, burnt bones are typically excluded from ZooMS analyses. However, there is uncertainty regarding the threshold temperature that prevents ZooMS analysis [54], and recent studies have shown successful retrieval of collagen peptides from incompletely burnt bones as determined by visual assessment [8, 55]. The five samples comprised in this study were visually characterised as burnt according to Stiner et al. [34] one fragment was assigned to Stage 2 (less than half carbonised: PG_003), three to Stage 3 (fully carbonised: PG_001, PG_004, PG_005), and one to Stage 4 (less than half calcined: PG_002). Although the MALDI-TOF analyses failed for all these specimens, we wanted to test whether high-resolution FTICRMS could provide a more productive alternative. The TFA-FTICR protocol yielded encouraging results, although taxonomic resolution remained low (S4 List of all identifications). The signal obtained with MALDI-FTICR is consistent with Bovidae/reindeer or Bovidae/Cervidae in three instances (PG-ICR-001, PG-ICR-002, PG-ICR-003), whereas two remains (PG-ICR-004, PG-ICR-005) indicate the presence of *Bos/Bison* biomarkers (peptide F [COL1α1 586–618]). The use of PRM LC–MS/MS and MALDI-CASI-FTICR analyses failed at detecting peptide A (COL1α2978–990), which prevented us from confirming these identifications (Data S4, list of all identifications).

4 | Discussion

This study compared traditional MALDI-TOF ZooMS analyses with high-resolution MALDI-FTICR mass spectrometry using Palaeolithic bone fragments with variable collagen preservation. The objective was to determine how MALDI-TOF and MALDI-FTICR can be integrated into a targeted ZooMS workflow aimed at optimising collagen extraction and spectral quality.

4.1 | Spectral Quality

We assessed spectral quality using two main criteria: [16, 56, 57] the resolution of the spectral data and the presence of diagnostic peptide markers.

4.1.1 | Spectral Resolution

The quality of a spectrum for PMF reflects the ease with which a peptide peak can be distinguished from background noise, contaminants and undesirable elements, such as polymers, trypsin, and matrix elements. The high number of peaks can obscure the detection of diagnostic peptide markers, especially when they are poorly ionised. As an example, in our study, peaks were 10 times more abundant in MALDI-FTICR spectra than in TOF spectra, which can confound the detection of diagnostic peaks. Poorly preserved peaks are less likely to be detected with MALDI-TOF, which paradoxically facilitates the detection of well-expressed markers. Consequently, the analysis of MALDI-FTICR spectra is more challenging and an automated process may reveal particularly useful in Palaeolithic contexts where collagen peaks tend to be poorly preserved [58].

4.1.2 | Peptide Markers Detection

Peptide marker detection is influenced by multiple factors, including collagen preservation within the bone, as well as the extraction protocol used, the reaction of each peptide, and the type of mass spectrometry. In our study, the retrieval of the nine target peptide markers showed some variation depending on the combinations tested in this study, leading to differences in taxonomic resolution. Similarly to other studies [7, 20, 36, 56] when some of these markers are missing, those that are most frequently absent are peptide A (COL1α2 978–990) and those with high masses (>*m/z* 2500). This trend was consistent across all protocols and both mass spectrometers and is probably related to collagen degradation. The chemical properties of those specific peptides and their interaction with lab reagents could also explain why they are usually less frequently recovered than other peptides, but this remains to be explored.

4.1.3 | Enhancing Peptide Marker A (COL1α2 978–990) With MALDI-FTICR MS

Although the use of intensity ratios did not improve the detection of peptide A (COL1α2 978–990), our results highlight three main conclusions regarding its detection in the studied assemblage:

i. The presence of a hydroxylated peak at *m/z* 1192.6796 is critical for the identification of *Bos/Bison* specimens; the unique presence of a *m/z* 1208.6745 peak is an insufficient criterion.

ii. The *m/z* 1166.6276 peak seems a reliable indicator of the presence of peptide A in reindeer, even when the hydroxylated *m/z* 1150.6327 peak is absent.

iii. Peptide A (COL1α2 978–990**)** tends to be more poorly ionised in reindeer than in *Bos/Bison*. When both *m/z* 1166.6276 and *m/z* 1208.6745 peaks are present, the former tend to be less intense, yet its detection (at a minimum s/n ratio of 3) is sufficient to confidently assign the sample to reindeer. However, it is critical to ensure the absence of contamination in this context.

4.1.4 | Contaminant Detection With MALDI-CASI-FTICR

The use of MALDI-CASI-FTICR facilitates the detection of contaminants. For instance, compared to the standard MALDI-FTICR analysis, the CASI mode improved the distinction of human keratin (e.g., [M + H] + 1192.63206, *m/z* 596.8199) from bovid collagen (e.g., [M + H] + 1192.67968, *m/z* 596.8435). These two components cannot be distinguished using MALDI-TOF due to its lower resolution.

4.2 | Tailoring Protocols and Instruments to the Study Material

For the same mass spectrometer, we observed substantial variability in the results depending on the extraction protocol in use. This suggests a strong link between protocol type and spectral quality.

4.2.1 | TOFMS

With MALDI-TOF, the three protocols tested gave very similar results, including the AmBic buffer, which, unlike HCl and TFA, does not involve bone demineralisation. This result was unexpected because the acid protocols should, in theory, increase the retrieval of collagen peptides [16, 56, 59]. However, it has also been shown that demineralisation can adversely impact protein survival [60]. Methodological factors may also explain this pattern. For instance, the acid HCl analysis was conducted a few months after the AmBic protocol, and storage in a freezer at −20°C may have altered collagen preservation [61]. Moreover, the TFA extractions were performed on smaller, single-spotted samples of bone powder (1–5 mg) which possibly impacted collagen recovery [16].

4.2.2 | FTICRMS

MALDI-FTICR MS provided variable results depending on the protocol used for collagen extraction (HCl or TFA). The results suggest that coupling TFA with MALDI-FTICR MS can increase the number of taxonomic identifications, even when collagen is highly degraded. It remains unclear which parameters directly affect the results given the large number of variables at play (e.g., sampling method, demineralisation time, the type of acid used and the filtration method; see Table S1).

4.3 | Optimised ZooMS Workflow

To optimise ZooMS identification rates both the extraction protocol and MS instrument can be tailored to the preservation state of the samples and the characteristics of the research question (Figure 5).

4.3.1 | Large-Scale Studies of Material With Unknown or Variable Collagen Preservation

Large-scale ZooMS studies usually involve sampling large numbers of bone fragments with limited or no information on collagen preservation. Because AmBic is less destructive than acid-based methods, the bone sample can be reused for further analyses; for this very reason, its use should be prioritised whenever possible. AmBic is particularly efficient at retrieving low mass peptides (<*m/z* 2500) such as peptide A (COL1α2 978–990), an important marker in ZooMS analyses. The HCl acid-based protocol can be used as a substitute when samples have failed

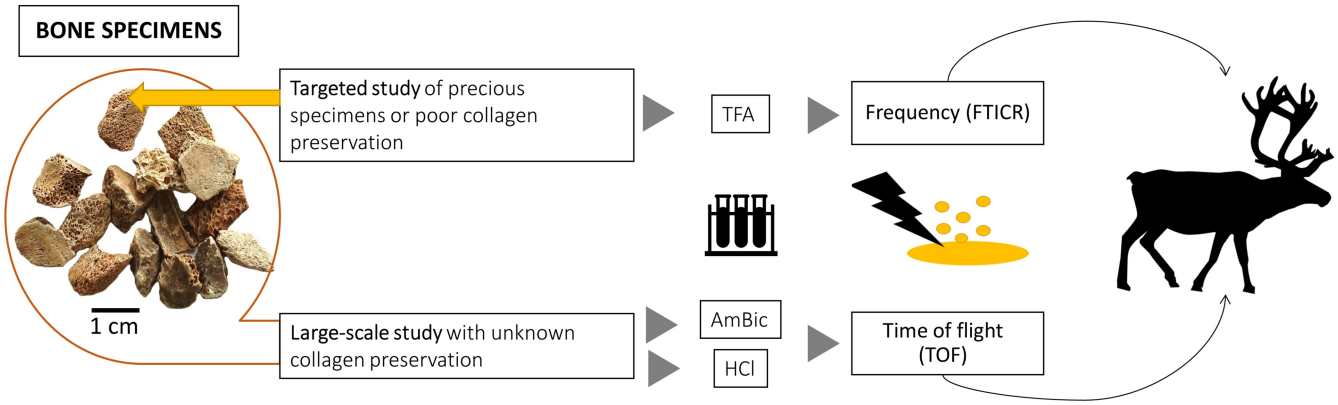

FIGURE 5 | New workflow that improves the use of MALDI-FTICR and MALDI-TOF mass spectrometers in ZooMS analyses. Targeted analysis focuses on a small number of remains showing poor collagen preservation that can benefit from a high-resolution MALDI-FTICR analysis. Large-scale studies are best carried out using MALDI-TOF with AmBic and HCl. These approaches can be combined with MALDI-FTICR for samples showing poor collagen preservation. [Colour figure can be viewed at wileyonlinelibrary.com]

or for poorly resolved identifications. Our study confirms that the HCl protocol combined with MALDI-TOF tends to produce more complete spectra—which, in our study, included most or all of the nine peptide markers we selected—resulting in better resolved identifications. Access to MALDI-TOF instruments is generally easier than for MALDI-FTICR, making it ideal for large-scale proteomic screening.

4.3.2 | Targeted Studies of Selected Specimens or Material With Known Poor Collagen Preservation

In contexts where the primary goal is to ensure maximal collagen extraction—for instance where sampling should be minimal (e.g., bone artefacts) [20, 55] or when collagen is poorly preserved (e.g., burnt bone)—the use of TFA and MALDI-FTICR should be favoured because this method seems more successful. Regarding peptide marker A (COL1α2 978–990), we recommend increasing the s/n ratio (up to five) to obtain more secure identifications. Our analyses also show that the MALDI-CASI-FTICR is a good complement to standard FTICR MS, as it is fast and effective at detecting contaminants and specific biomarkers, and appears to be as efficient as PRM LC–MS/MS analyses. Finally, MALDI-CASI-FTICR is a useful tool for pre-screening large sets of samples with a standard ZooMS protocol and as quality control to verify the exact mass of key discriminating peptides.

4.4 | Future Directions

Despite some limitations, our results show that MALDI-FTICR provides a convenient alternative that complement the ZooMS MALDI-TOF workflow, especially when combined with the TFA protocol. There are several ways in which the potential of the approach can be enhanced in the future.

4.4.1 | Sample Size

For future applications, we recommend increasing the quantity of sampled bone material from 5 to 20 mg, particularly when collagen preservation is likely to be variable. These larger samples can then be examined using multiple approaches to collagen extraction. Sampling bone chunks rather than bone powder may also improve collagen retrieval [12], although this remains to be verified with MALDI-FTICR.

4.4.2 | The Use of Replicates

To optimise the ZooMS workflow, we assessed whether high-resolution MALDI-FTICR spectra could be obtained without using triplicates for degraded samples [10], thereby minimising both costs and processing time. Our work showed that MALDI-FTICR outperformed MALDI-TOF when the protocol (TFA) was combined with single spotting (SI 4, List of all identifications). Because MALDI-TOF is known to perform better with triplicate spotting—an observation further supported by our study—we predict that the use of replicates with MALDI-FTICR should further improve precision in taxonomic identification. Alternatively, the demineralisation solutions kept through the TFA protocol can be used to minimise protein loss. In our study, they provided very few collagen peaks, which explain why they were excluded from our comparisons. However, the spectra are not always blank, which might prove useful when examining precious or degraded samples.

4.4.3 | Time and Cost Effectiveness

ZooMS is increasingly being used in archaeological research largely due to its accessibility and low costs. The use of MALDI-FTICR permits rapid analysis without necessitating additional expertise in data processing, thereby facilitating its integration into the ZooMS workflow. While MALDI-FTICR provides higher resolution data, analytical costs are higher than for MALDI-TOF, but lower than for LC–MS/MS. To minimise expenses and implement an accessible new ZooMS workflow, we did not prioritise measuring replicates. However, if MALDI-FTICR is integrated into a targeted analysis involving smaller sample sets, the use of replicates is feasible while maintaining relatively low costs.

4.4.4 | Burnt Material

Preliminary results are encouraging as they show that MALDI-FTICR can successfully be used to identify burnt bone. Additional experimental work on burning temperature [34], combined, for instance, with FTIR techniques (Fourier-transform infrared spectroscopy) [52, 62] is needed to fully explore the potential of this approach in ZooMS analysis.

5 | Conclusion

Our study shows that MALDI-TOF performs better when combined with triplicate spotting as applied with the AmBic and HCl protocols. MALDI-TOF is particularly effective when collagen is well preserved and when assessing large quantities of material as the spectral analysis is simplified. However, MALDI-FTICR used with the TFA protocol outperforms all other methods in terms of collagen retrieval. This may be critical when collagen is highly degraded (including when the specimen has been exposed to heat). MALDI-FTICR thus emerges as a robust complementary tool to MALDI-TOF, especially to enhance poorly resolved MALDI-TOF taxonomic identifications. In addition, MALDI-CASI-FTICR can also be used for targeted analyses in contexts of poor collagen preservation and for ascertaining the presence of specific taxa in faunal assemblages. MALDI-CASI-FTICR can achieve comparable results as PRM LC–MS/MS in a fraction of the time. Our study also demonstrates that combining several mass spectrometry techniques to analyse poorly preserved samples can enhance the resolution and robustness of taxonomic attributions.

Author Contributions

Pauline Raymond: investigation, writing – original draft, writing – review and editing, formal analysis, conceptualization, methodology, resources, validation. **Karen Ruebens:** supervision, writing – review

and editing, conceptualization, validation. **Fabrice Bray:** investigation, funding acquisition, writing – review and editing, methodology, formal analysis, resources, validation, conceptualization, supervision. **Jean-Christophe Castel:** data curation. **Eugène Morin:** writing – review and editing, data curation. **Foni Le Brun-Ricalens:** data curation. **Jean-Guillaume Bordes:** data curation. **Christian Rolando:** writing – review and editing, methodology, resources, funding acquisition, validation, conceptualization, supervision. **Jean-Jacques Hublin:** supervision, resources, funding acquisition.

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Data Availability Statement

The ZooMS spectra (in mzxml format) and the database comprising the full list of taxonomic identifications are available on Zenodo: https://doi.org/10.5281/zenodo.13151059.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.10019.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.