Proteomics
and high resolution mass spectrometry for identification, dating and bones isotopic ratio of archaeological and paleontological bones

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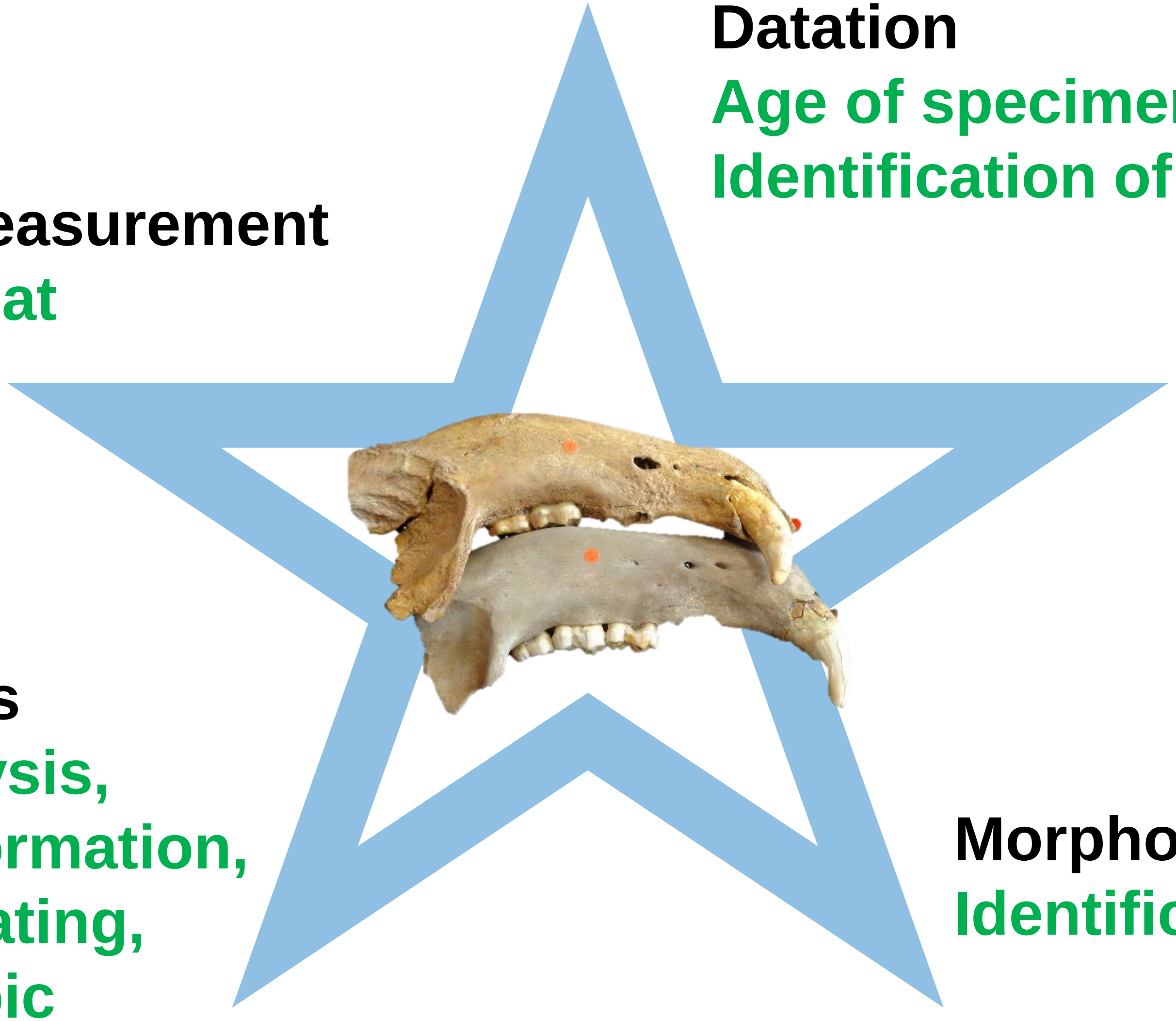
Miniaturization for Synthesis, Analysis & Proteomics UAR 3290

EU_FT-ICR_EUS, Monday 12th December 2022

University of Lille, FST

Introduction



Datation
Age of specimen,
Identification of geological era

Isotopic ratio measurement
Diet, food they eat

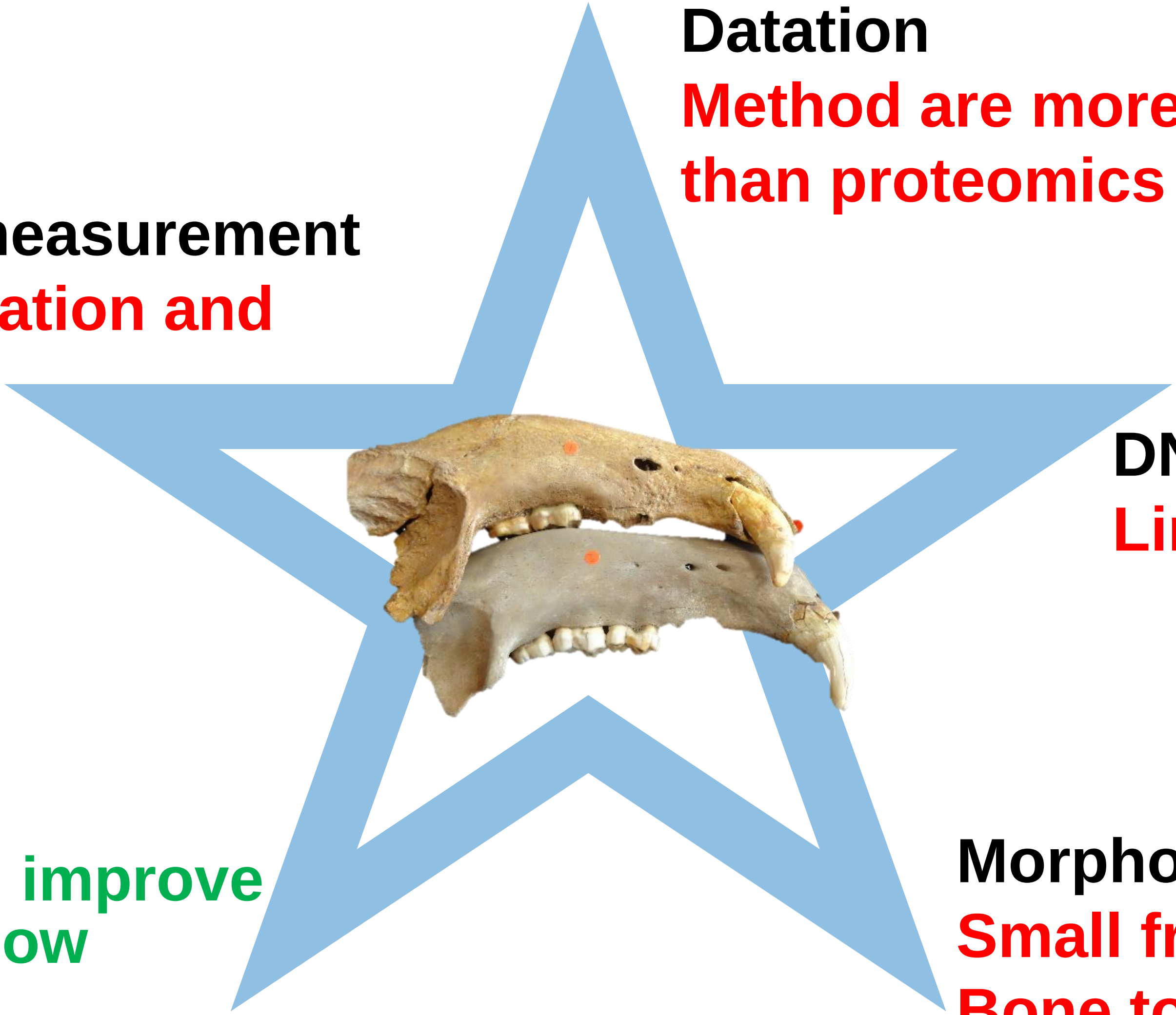
DNA analysis
DNA sequence
Phylogenetic reconstruction

Protein analysis
Proteome analysis,
Diagenesis information,
Approximate dating,
Possible Isotopic measurement

Morphology
Identification of taxa

Maxillary form cave bear
Ursus speleaus

Introduction



Datation
Method are more destructive than proteomics

Isotopic ratio measurement
Specific preparation and analysis

DNA analysis
Limited by DNA degradation

Protein analysis
Need to improve proteomic workflow

Morphology
Small fragment
Bone tools

Problematic

- **Identification of extinct species** and **bones unidentifiable by morphology:**

  Bone tools made by prehistoric Humans, burned and small fragments


Femur form *Mammuthus primigenius*

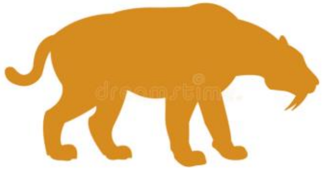

Small fragments


Bone tools form Scladina cave

- **Phylogenetic** reconstitution:

  Fascinating species


Cave bear
(Ursus spelaeus)

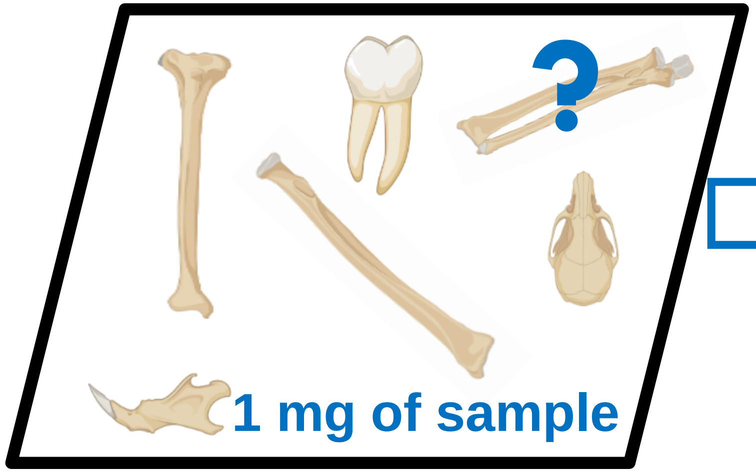

Saber-toothed cat
***(**Homotherium latidens)*

- **Estimation of datation** by proteomics:

  Find other markers that can be correlated with deamidation

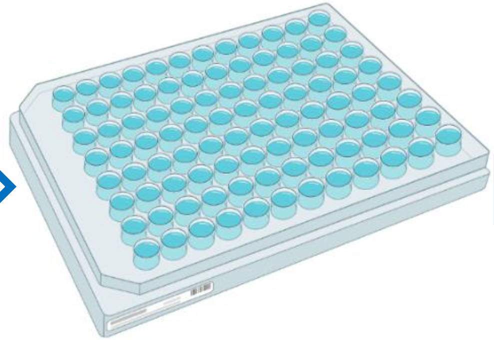
High throughput proteomics workflow

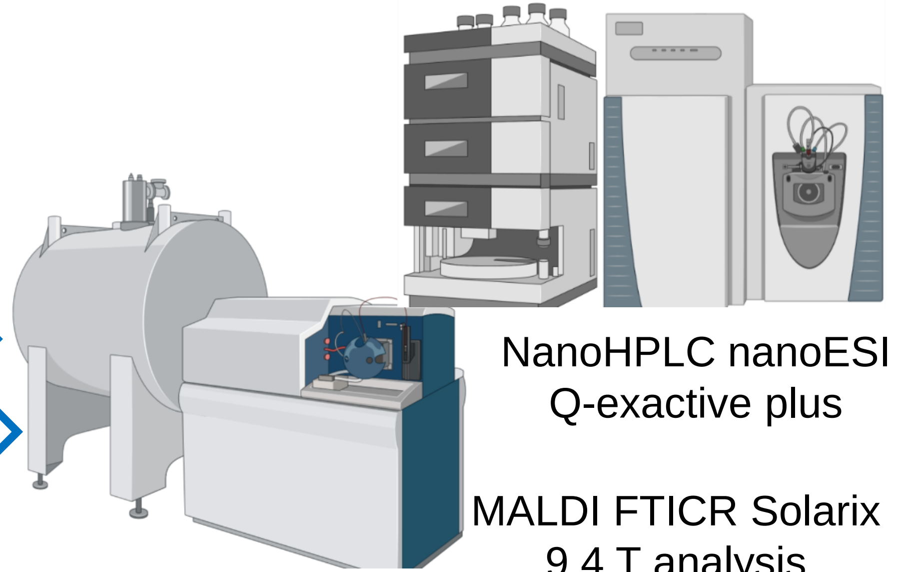
310 bones from Holocene up to Pleistocene



1 mg of sample

All steps in 96 well plate

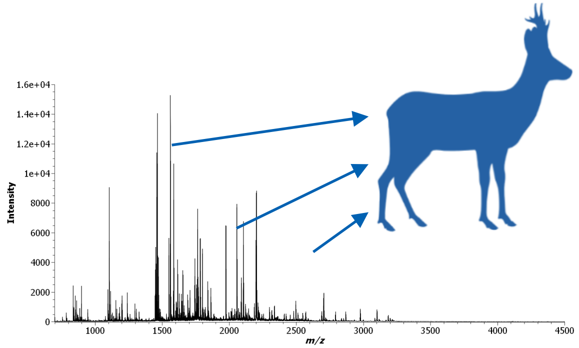




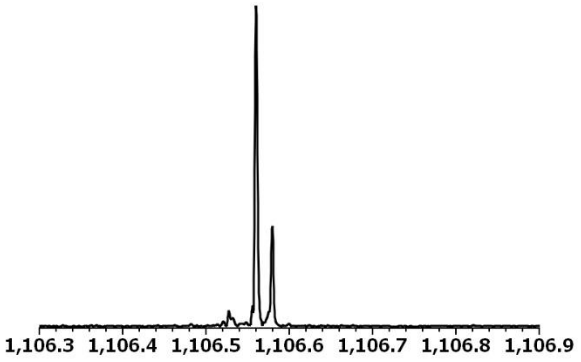
NanoHPLC nanoESI
Q-exactive plus

MALDI FTICR Solarix
9.4 T analysis

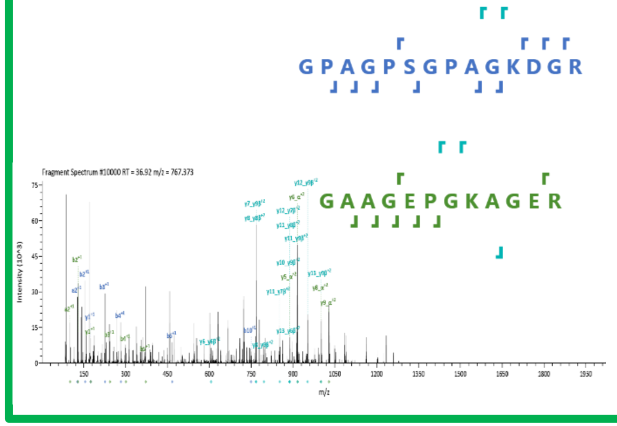
Identification of bone



Deamidation rate

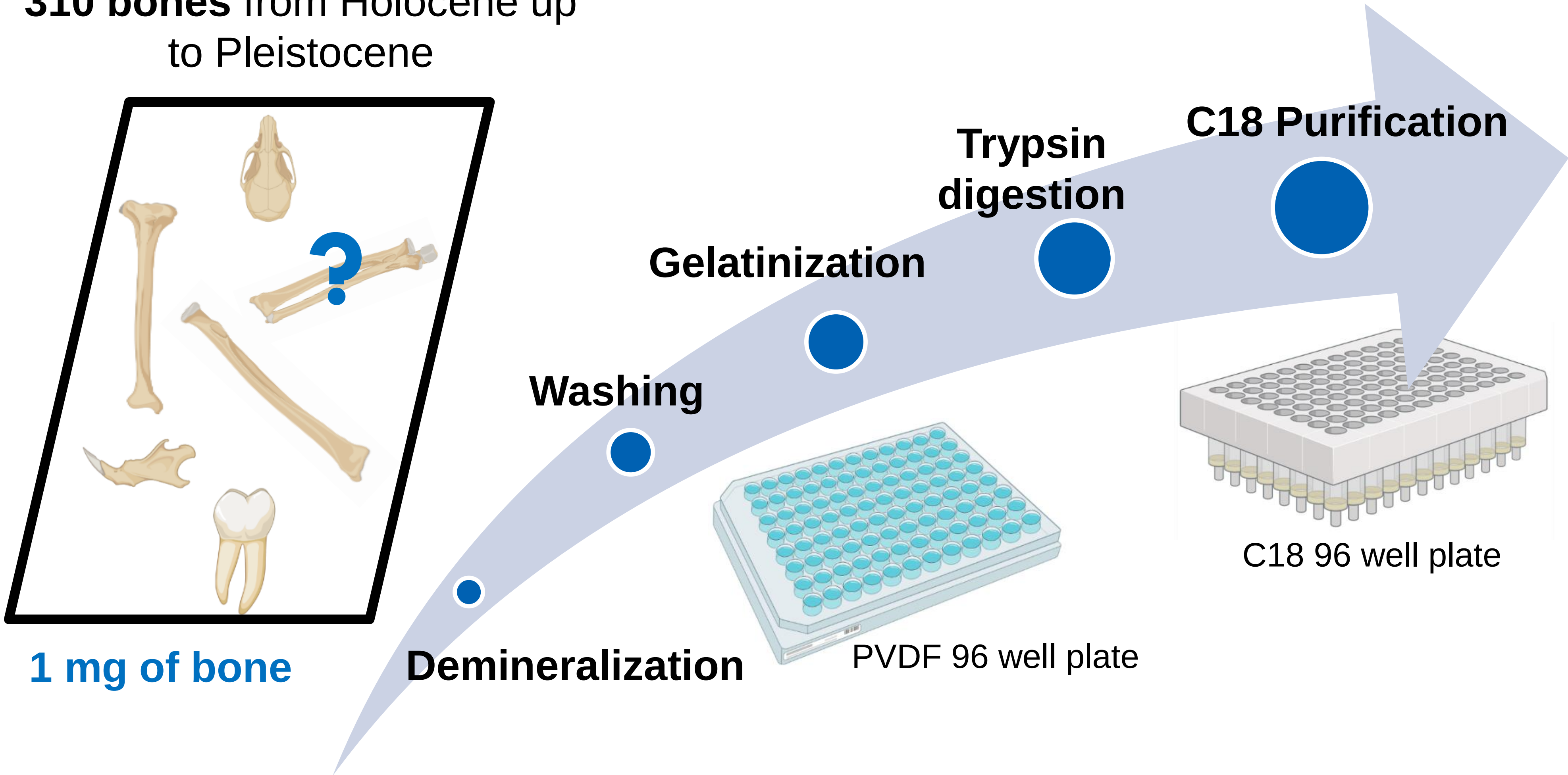


Crosslink quantification

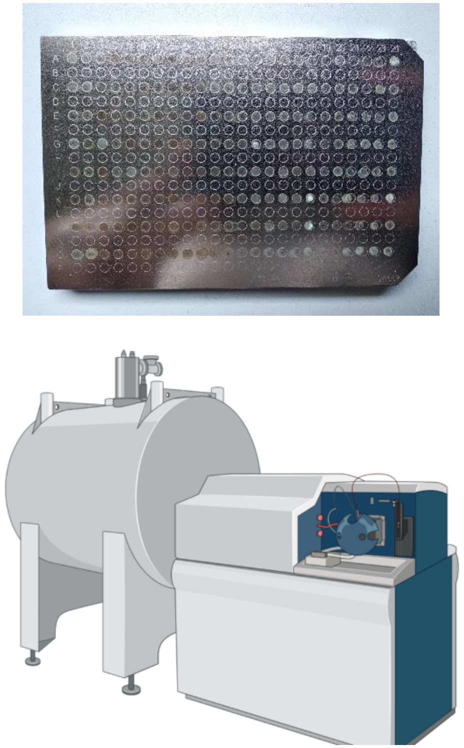


Detailled high throughput proteomics workflow

310 bones from Holocene up to Pleistocene



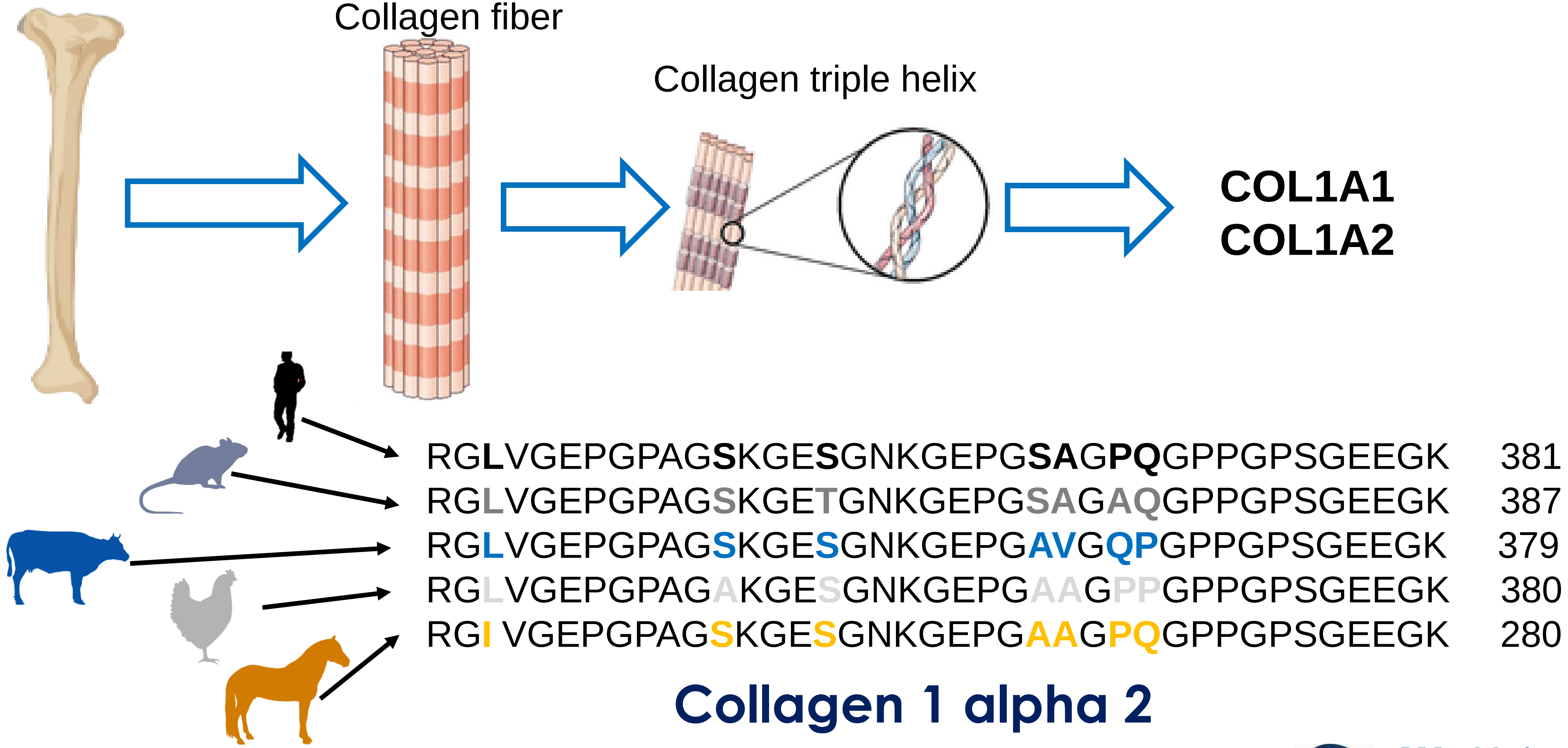
1 mg of bone



MALDI FTICR Solarix
9.4 T analysis

Fabrice Bray, Isabelle Fabrizi, Stéphanie Flament, Jean-Luc Locht, Pierre Antoine, Patrick Auguste, Christian Rolando, (**2022**) Robust high throughput proteomics identification and deamidation quantitation of extinct species up to Pleistocene with Ultrahigh Resolution MALDI-FTICR Mass Spectrometry, *Analytical Chemistry*, submitted for publication

Identification of taxa (family, gender, species) with specific markers

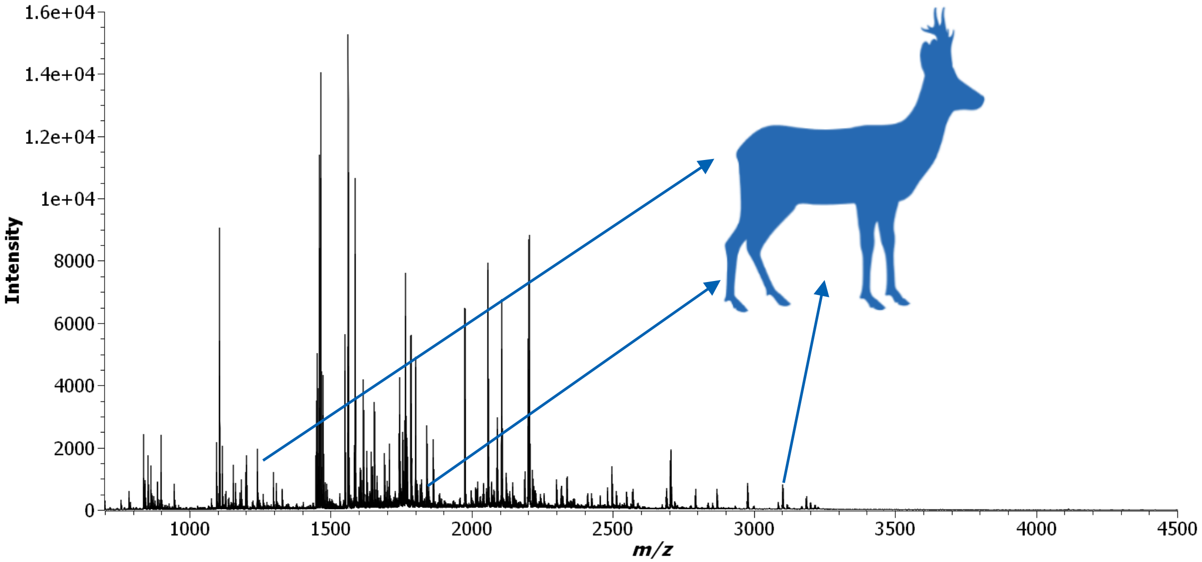


RG**L**VGEPGPAG**S**KGE**S**GNKGEPG**SA**G**PQ**GPPGPSGEEGK	381
RGLVGEPGPAGSKGETGNKGEPGSAGAQGPPGPSGEEGK	387
RGLVGEPGPAGSKGESGNKGEPGAVGQPGPPGPSGEEGK	379
RGLVGEPGPAGAKGESGNKGEPGAAGPPGPPGPSGEEGK	380
RGI VGEPGPAGSKGESGNKGEPGAAGPQGPPGPSGEEGK	280

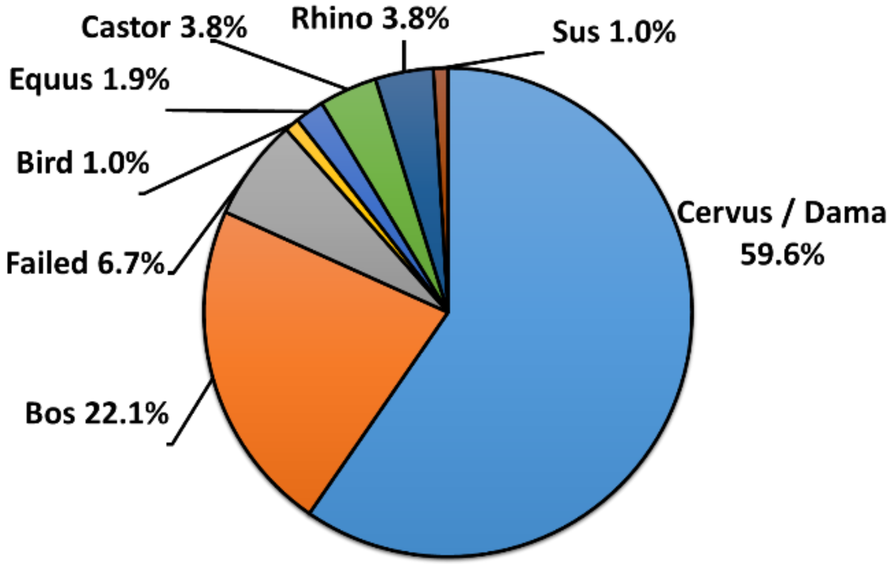
Collagen 1 alpha 2

Buckley, M., Collins, M., Thomas-Oates, J., & Wilson, J. C. (2009). Species identification by analysis of bone collagen using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry, 23(23), 3843-3854.

Identification of bones by MALDI FTICR MS from Caours site (France)



MALDI FTICR spectrum of *Cervus elaphus* (roe deer)

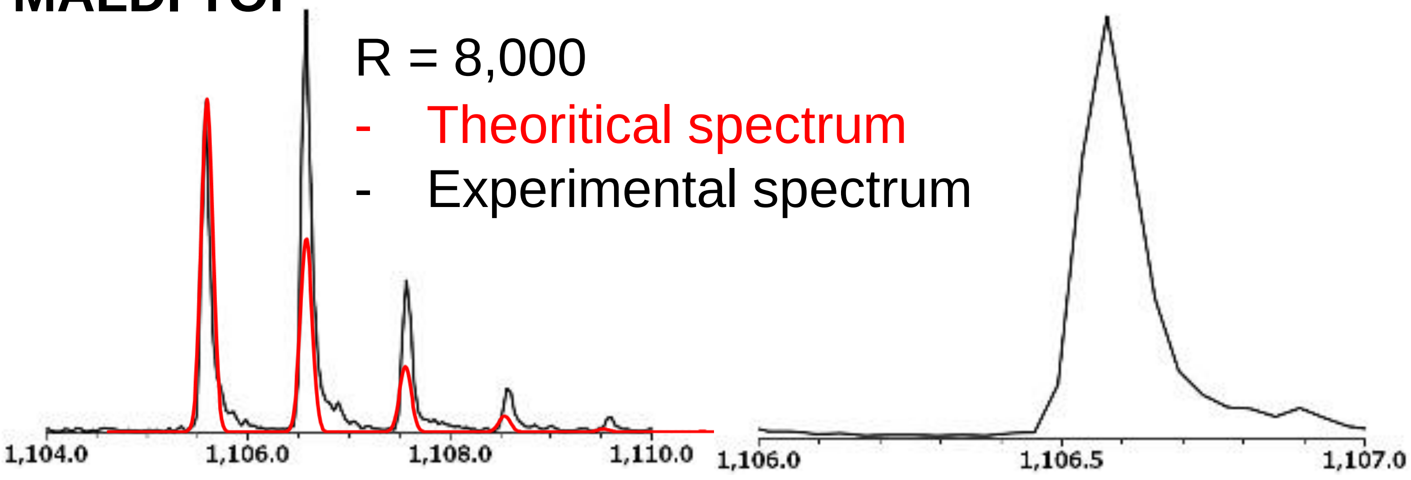


Number of identified with morphological identification and MALDI FTICR MS identification

- **104 bones 130,000 years old from the Caours site** (Somme, France) were analyzed by MALDI FTICR
- An overall success rate of **93%** was achieved by the MALDI FTICR MS analysis
- **55%** of the bones are not **identified** by paleontologists by their morphology
- **91% of unknown bones** were **identified by MALDI FTICR MS analysis**

Comparison MALDI-TOF vs MALDI FTICR

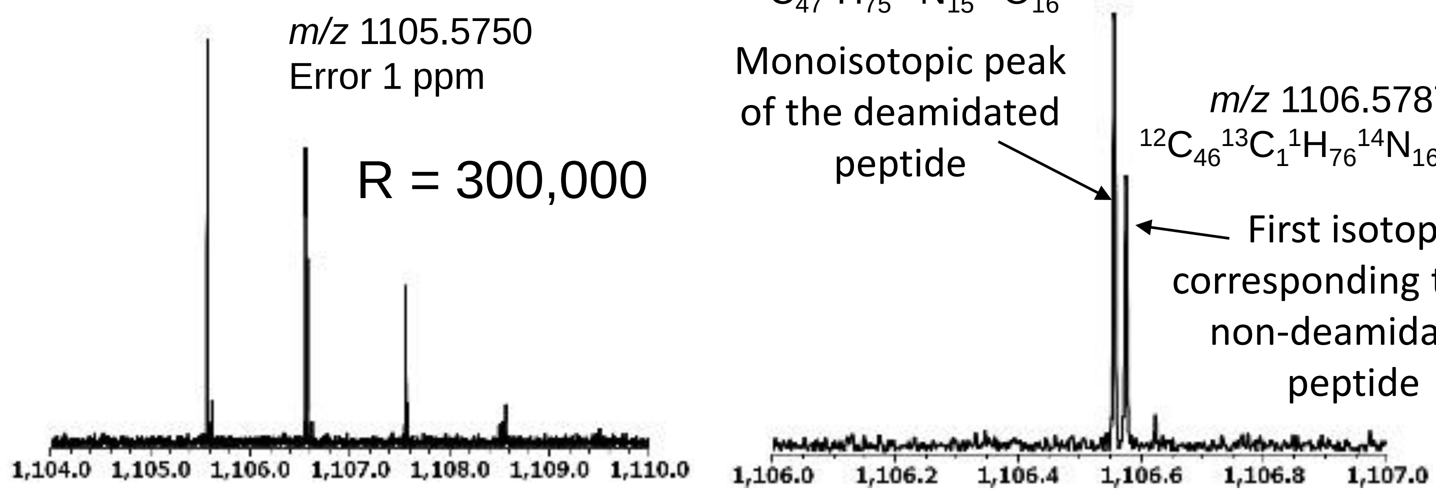
MALDI TOF



R = 8,000

- Theoritical spectrum
- Experimental spectrum

MALDI FTICR



m/z 1105.5750
Error 1 ppm

R = 300,000

m/z 1106.5590
$^{12}C_{47}{}^{1}H_{75}{}^{14}N_{15}{}^{16}O_{16}$

Monoisotopic peak of the deamidated peptide

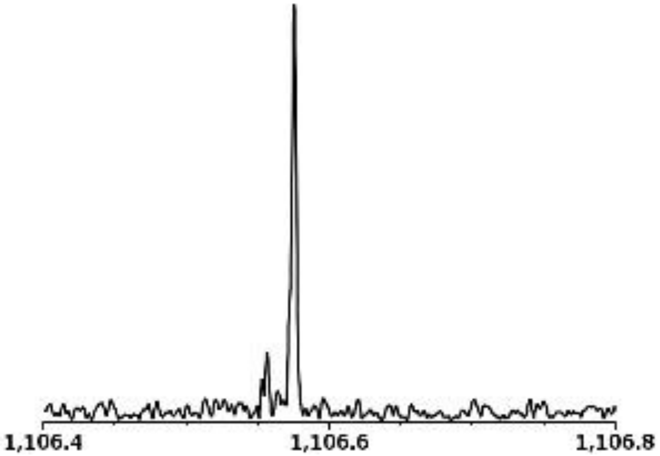
m/z 1106.5787
$^{12}C_{46}{}^{13}C_{1}{}^{1}H_{76}{}^{14}N_{16}{}^{16}O_{15}$

First isotope corresponding to the non-deamidated peptide

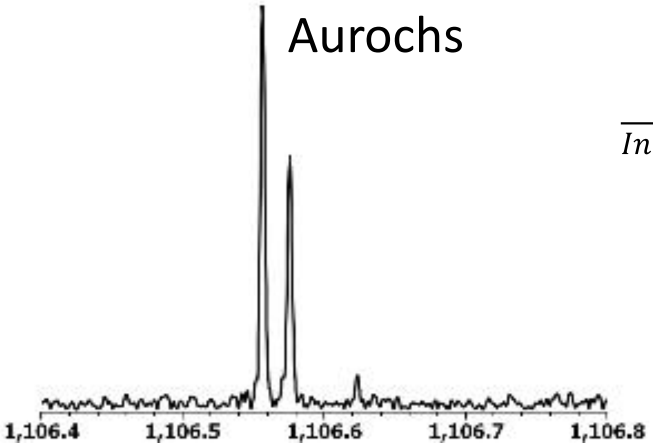
- *Bos primigenius Taurus (aurochs)* bone digestion (sample Ca 101)
- Study of GVQGP^{ox}PGPAGPR sequence: the universal peptide in mammalian collagen
- **Deamidated peptide** GVQ^{dem}GP^{ox}PGPAGPR is present in bone digestion
- **2M acquisition** is sufficient to measure precisely the deamidation
- FTICR allows to observe a mass **difference of 0.019 Da** (deamidation)
- Error **less than 1ppm**

Evaluation of the deamidation by MALDI FTICR MS

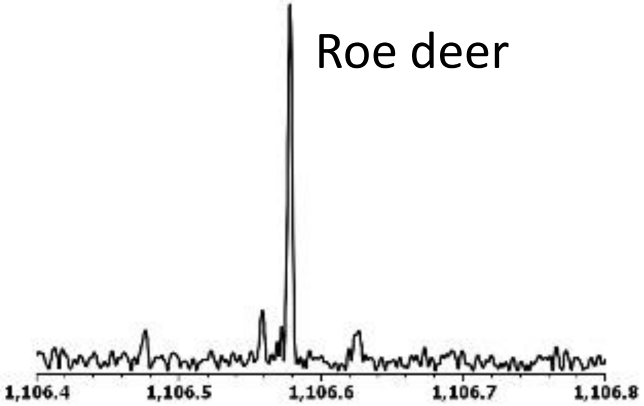
modern *Bos Taurus*



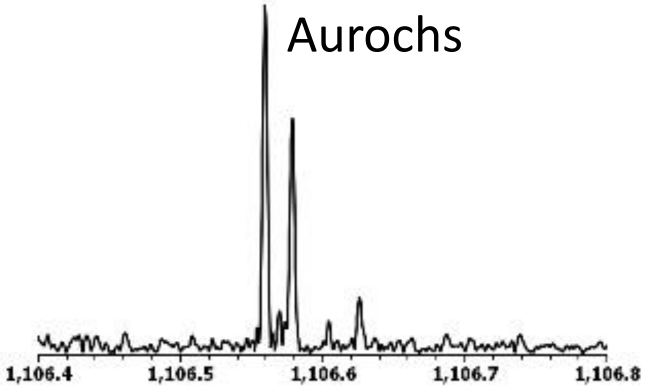
Bos primigenius Ca 101



modern *Cervus elaphus*



Bos primigenius Ca 102



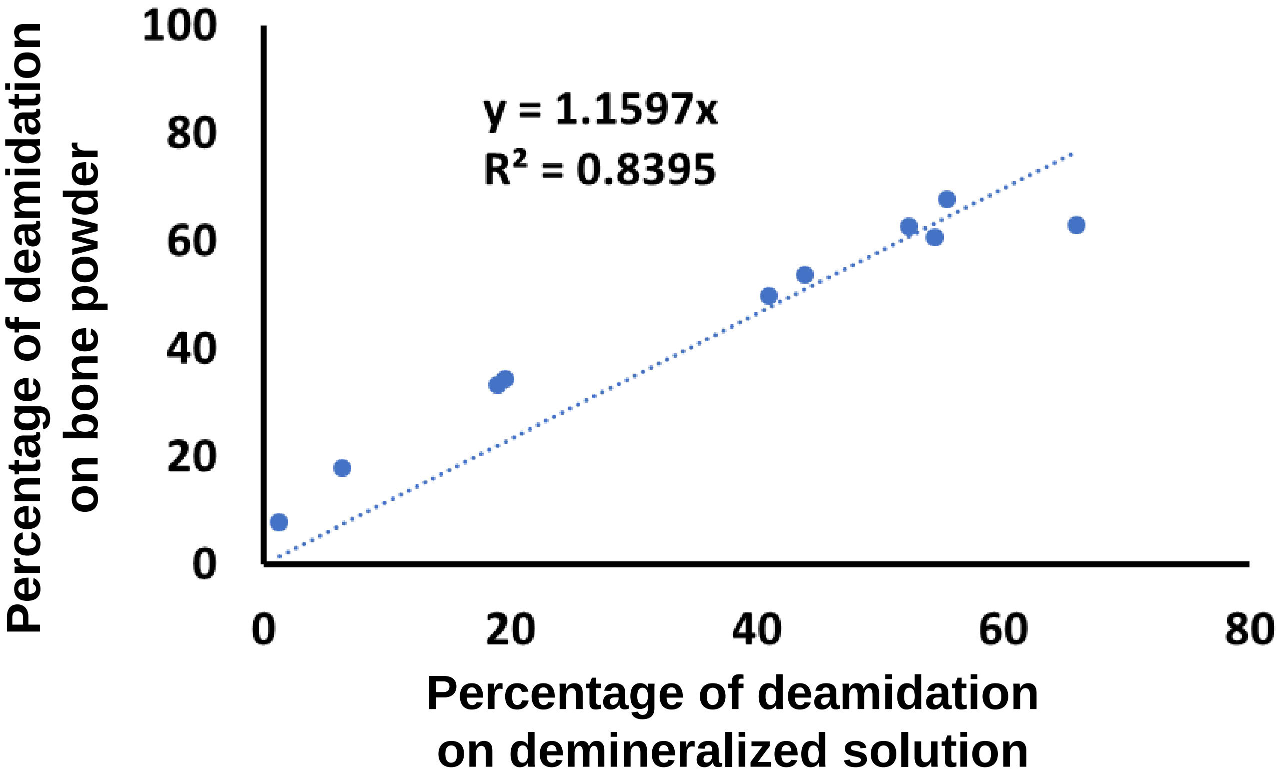
- Glutamine deamidation is **related to chronology**

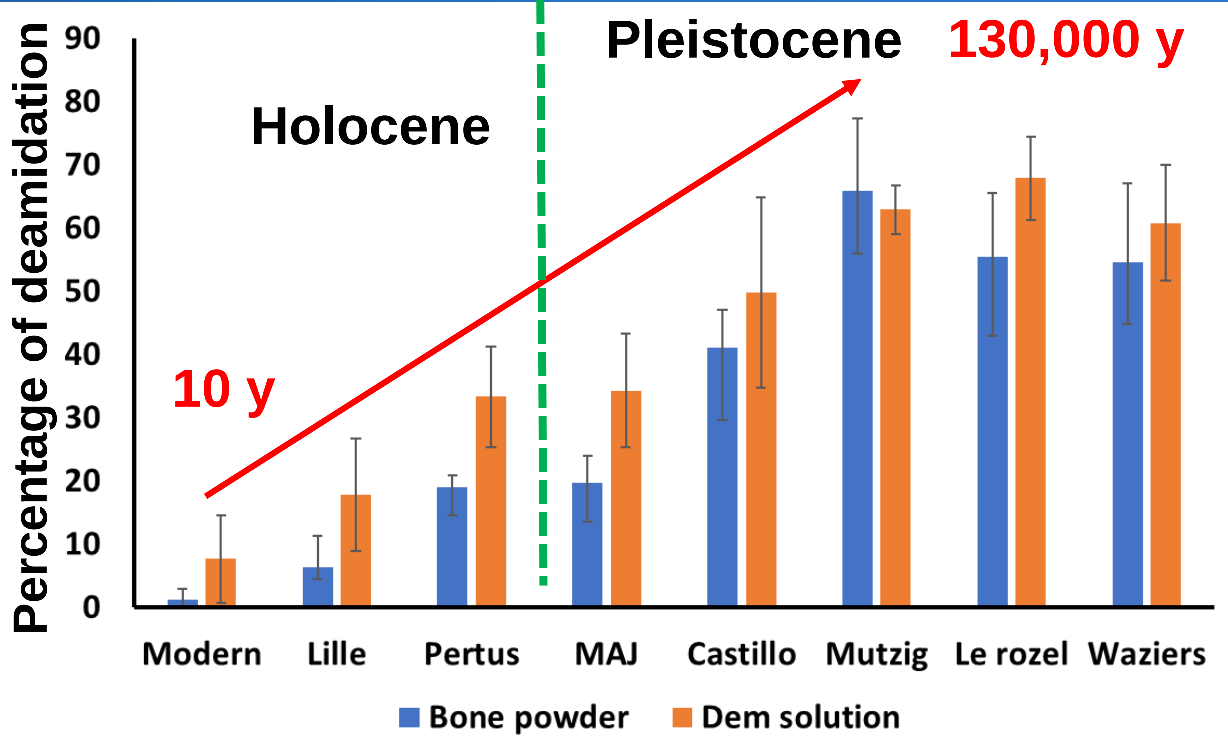
$$\frac{Intensity\ of\ deamidated\ peptide}{Intensity\ of\ deamidated\ peptide + intensity\ of\ non\ deamidated\ peptide} \times 100$$

- *Bos Taurus* modern sample deamidation was **0.6% +/- 0.7%** and **4.4% +/- 1.2** for *Cervus elaphus* modern sample
- Caours101 deamidation was of **39.4% +/- 2.4%** and **40.7% +/- 2.9%** (Caours 102)
- The **average of deamidation** for Caours site (130,000 years) MALDI-FTICR was **44.3%** +/- 8.4%

Chowdhury, M. P., & Buckley, M. (2022). Trends in deamidation across archaeological bones, ceramics and dental calculus. *Methods*, *200*, 67-79.

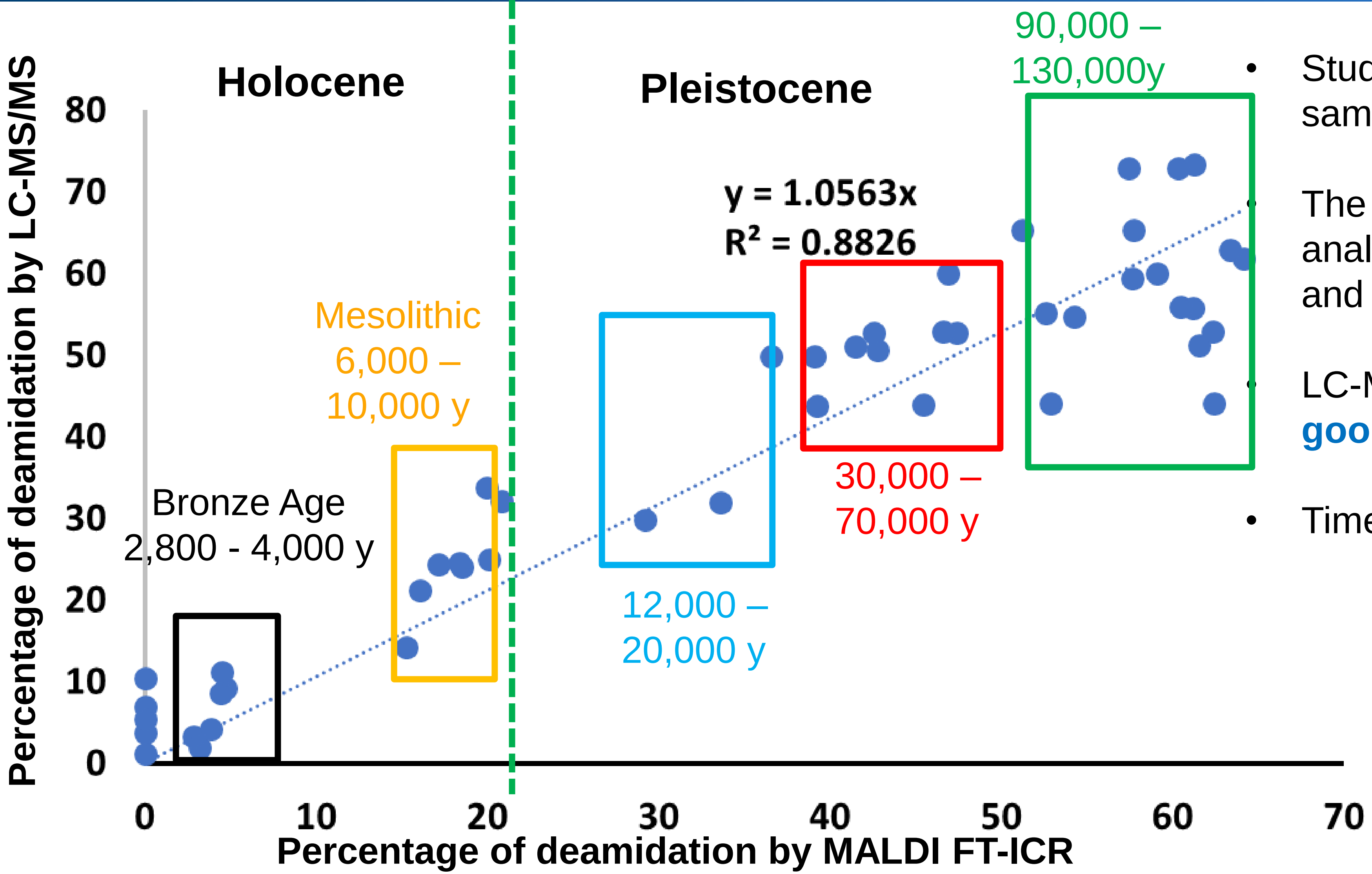
Evaluation of demineralization on the deamidation rate by MALDI FTICR MS





- Study of samples from **Holocene** (35 samples) and **Pleistocene** (61 samples) form **10 different sites**
- Deamidation rate of bone powder and demineralized solution shows a **good correlation** (**R = 0.83**)
- Demineralization solution with **TFA induce deamidation**

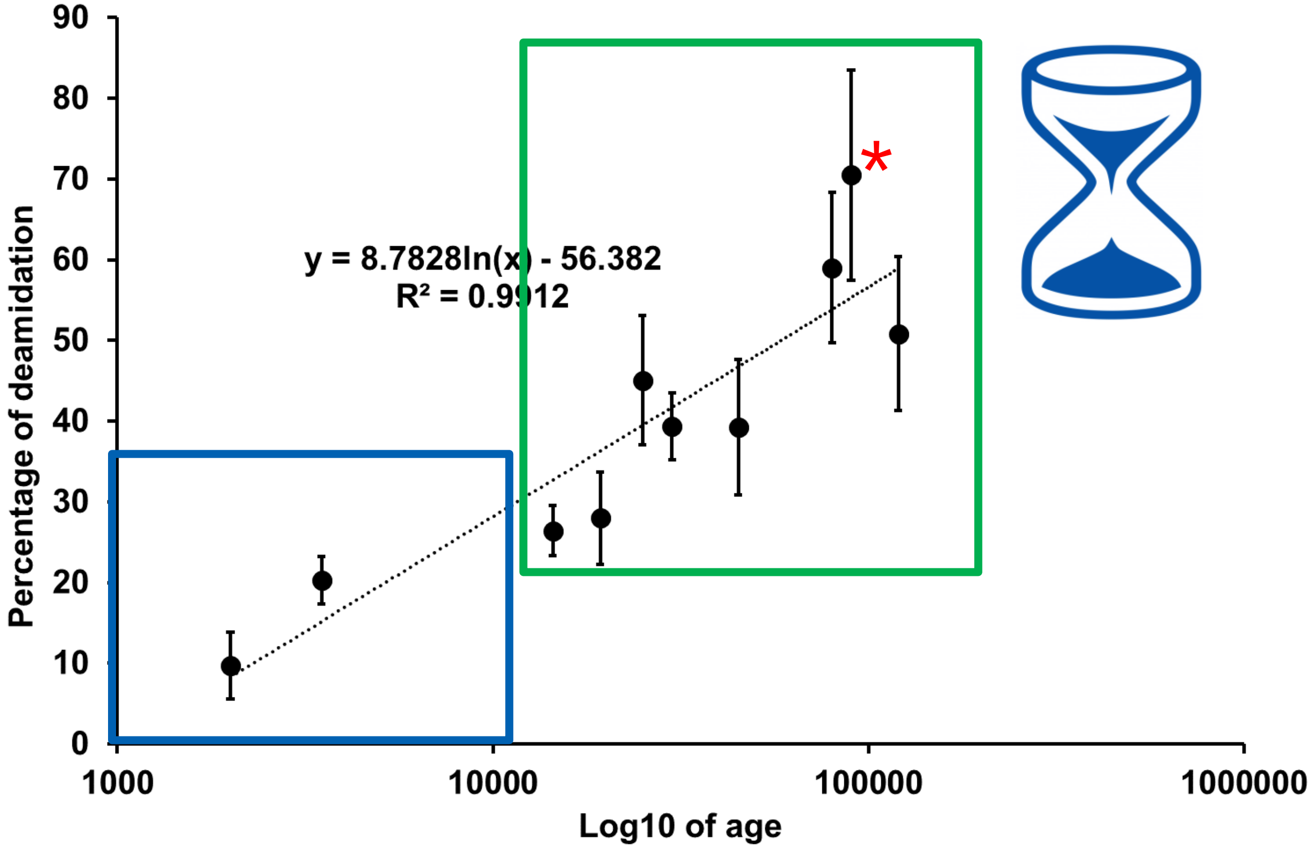
Correlation between MALDI FTICR and LC-MS/MS



- Study of samples from Holocene (20 samples) and Pleistocene (30 samples)
- The analysis of deamidation with the 2 analytical methods MALDI FTICR MS and LC-MS/MS
- LC-MS/MS and MALDI FT-ICR shows a **good correlation** (**R = 0.88**)
- Time analysis: **LC-MS/MS 120 min**
  MALDI FTICR 2 min

Bray, F., Flament, S., Abrams, G., Bonjean, D., Rolando, C., Tokarski, C., & Auguste, P. (2022). Extinct species identification from late Middle Pleistocene and earlier Upper Pleistocene bone fragments and tools not recognizable from their osteomorphological study by an enhanced proteomics protocol. Archaeometry.

Dating estimation by MALDI FTICR MS

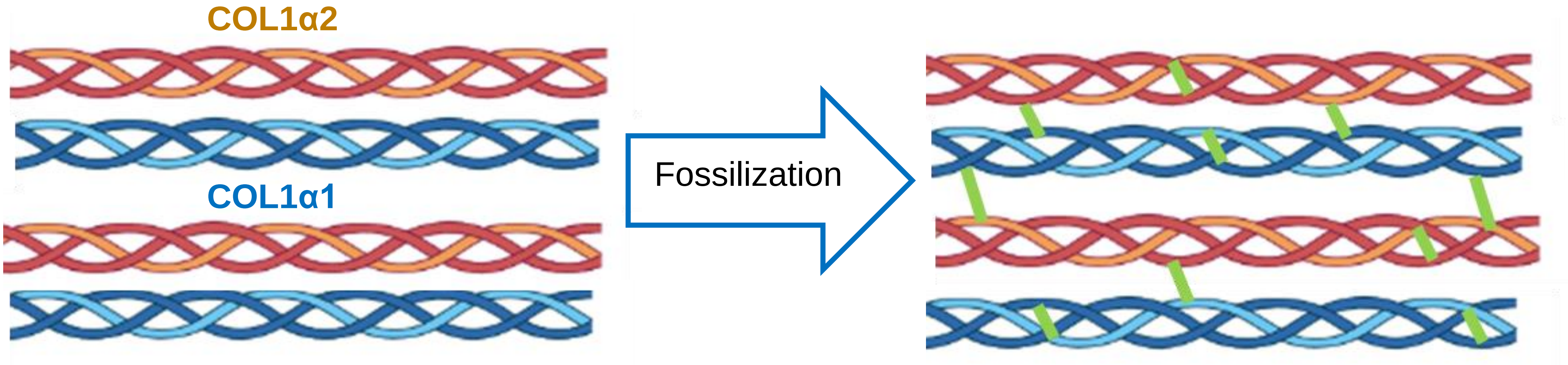


- **310 bones** from **Holocene** (11,000 years ago - present, France) up to **Pleistocene** (120,000 years ago, Europe)
- The rate of deamidation of the peptide at mass *m/z* 1,105.5590 showed a **significant difference between the sites**
- Deamidation rate of modern samples 1.2% +/-1%
- * Corresponding to bones from Mutzig (France) site where the soil is very acidic

- The archaeological **conservation and environmental** context are different between the sites and can **induce variability** both in and among samples
- It is interesting to look for **other proteomics markers that correlate with age**

Crosslinking of proteins during fossilization

- The **fossilization transform** tissus
- **Oxidative changes affect all structures** with a proteinaceous scaffold
- Raman spectroscopy studies on fossils suggest the **presence of crosslink**

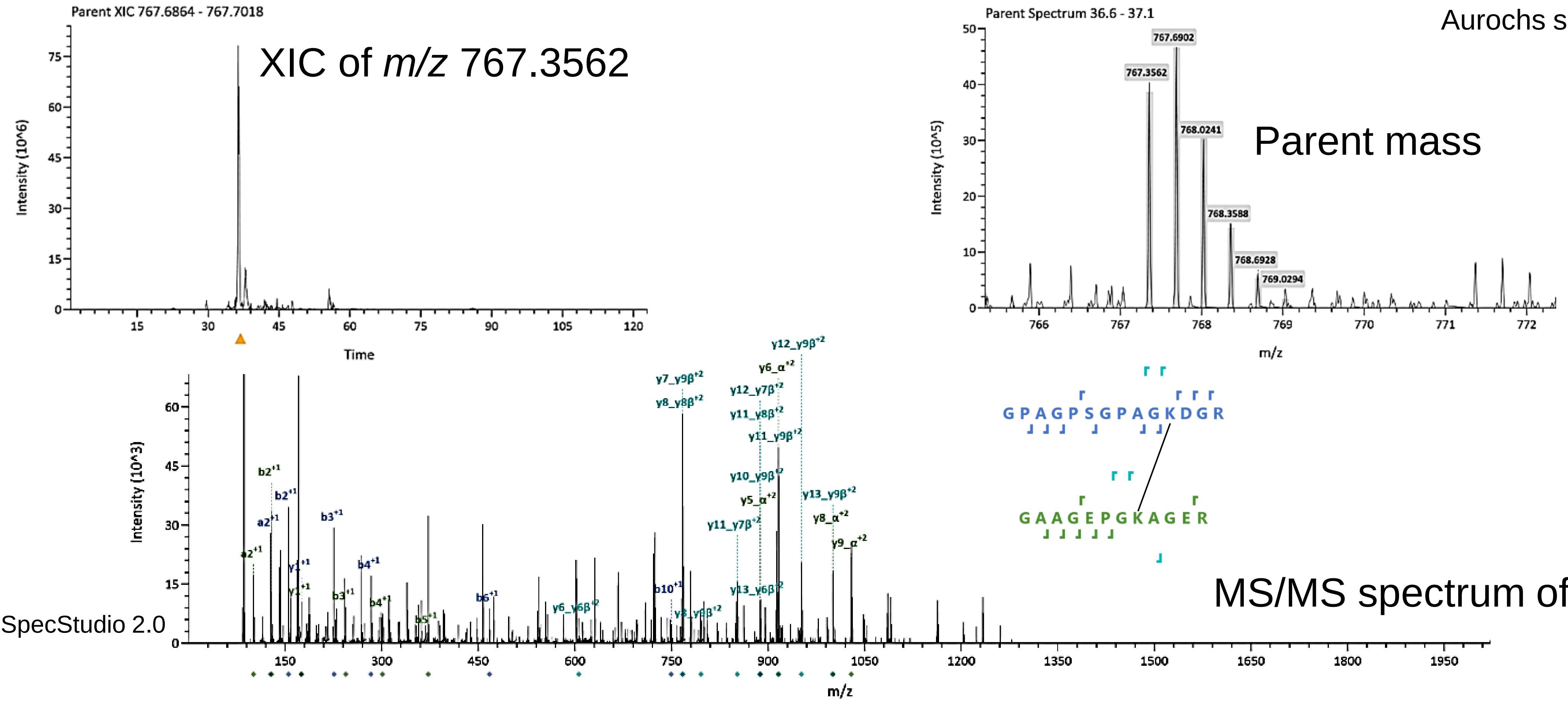


- **Link between fossilization, crosslink and age ?**

Wiemann, J., Fabbri, M., Yang, T. R., Stein, K., Sander, P. M., Norell, M. A., & Briggs, D. E. (2018). Fossilization transforms vertebrate hard tissue proteins into N-heterocyclic polymers. *Nature Communications*, *9*(1), 1-9.

DHLNL crosslinking by nanoLC-MS/MS Orbitrap

Aurochs sample from Caours site



XIC of *m/z* 767.3562

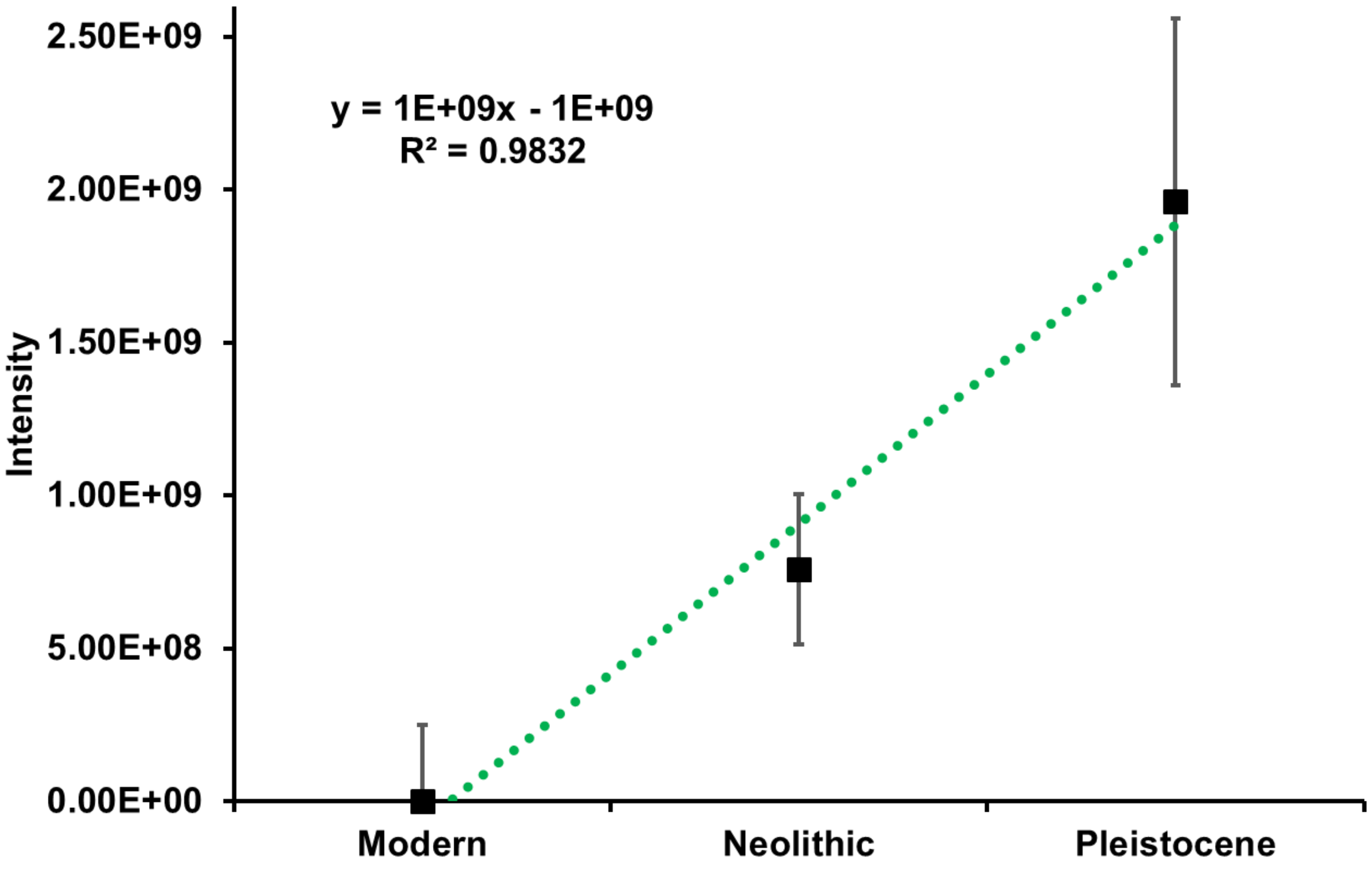
Parent mass

MS/MS spectrum of *m/z* 767.3562

MassSpecStudio 2.0

- Dehydroxylysinorleucine **DHLNL crosslink was studied**
- The peptide at mass *m/z* 767.3562 with 3 charges was quantified
- Crosslink of **COL1α1** peptide GPAGPSGPAG<u>K</u>GR with **COL1α2** peptide GAAGEPG<u>K</u>GER

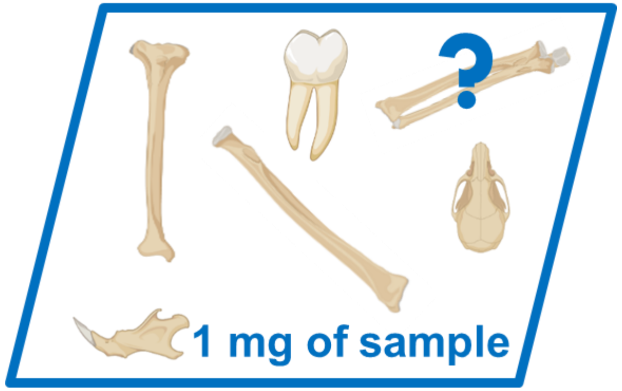
Quantification of crosslinking

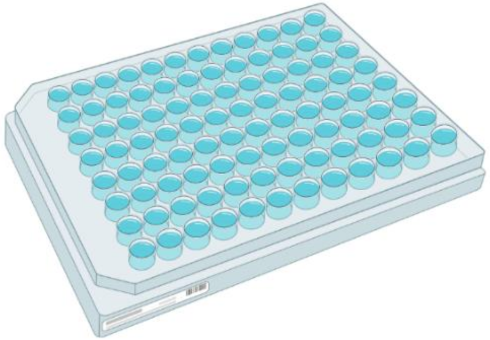


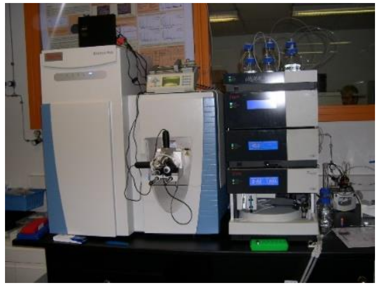
- Injection of 1 µg
- Analysis of *Bos* samples
- 3 samples for each period

- Collagen **crosslinking increases** as bone **fossilization** progresses.
- A **linear correlation** between modern Holocene and Upper Pleistocene samples was observed
- This **linear correlation** **is potentially more accurate than the logarithmic one for deamidation**

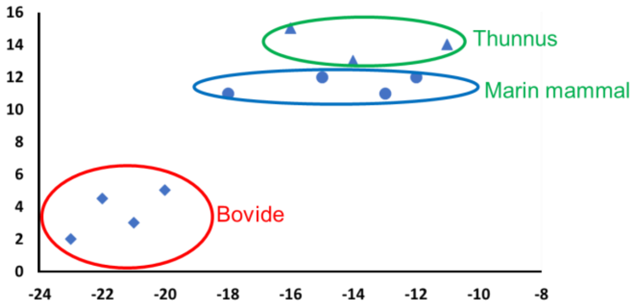
Isotopic ratio by nanoLC-MS/MS orbitrap







nanoLC-MS/MS
Colonne C18
MS & MS/MS **70K**
HCD 50 eV
Gradient 140 min

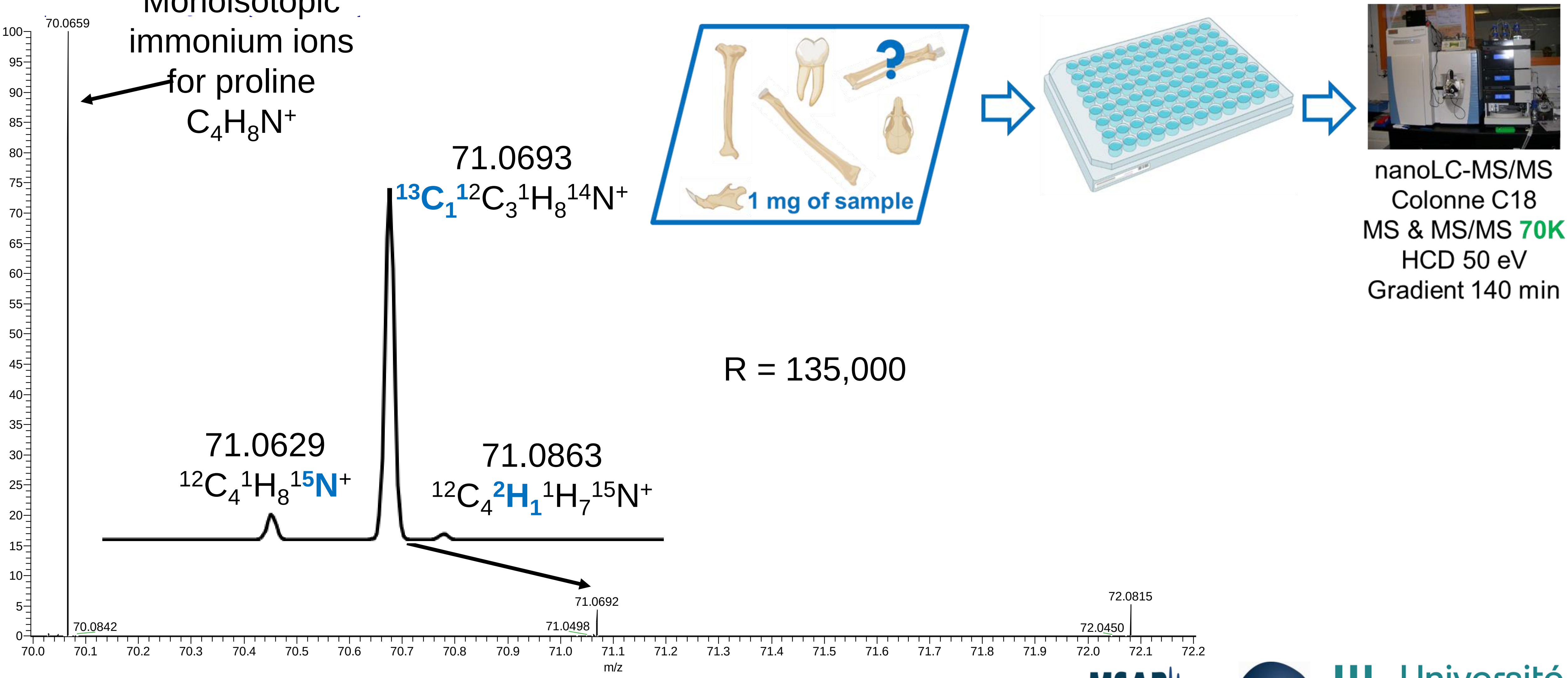


Bivariate plot of **δ^{13}C and δ^{15}N**

- Study of the **δ^{13}C, δ^{15}N and δ^{2}H** isotopic ratio
- **Broad *m/z* window** for precursor ion isolation followed by fragmentation
- Analysis of **MS/MS spectra obtain with HCD (50 NCE)**
- Focus on **immonium ion**
- **Diet Information** of specimen

Gharibi, H., Tchernobrovkine, A. L., Eriksson, G., Ata Saei, A., Timmons, Z., Kitchener, A. C., Kalthoff, D. C., Lidén, K., Makarov, A. A, & Zubaverv, R. A. **(2022)**. Abnormal (Hydroxy)proline Deuterium Content Redefines Hydrogen Chemical Mass. . *Am. Chem. Soc.* 2022, 144, 6, 2484–2487.

MS/MS spectrum with proline immonium



Monoisotopic immonium ions for proline $C_4H_8N^+$

71.0693 $^{13}C_1{}^{12}C_3{}^{1}H_8{}^{14}N^+$

71.0629 $^{12}C_4{}^{1}H_8{}^{15}N^+$

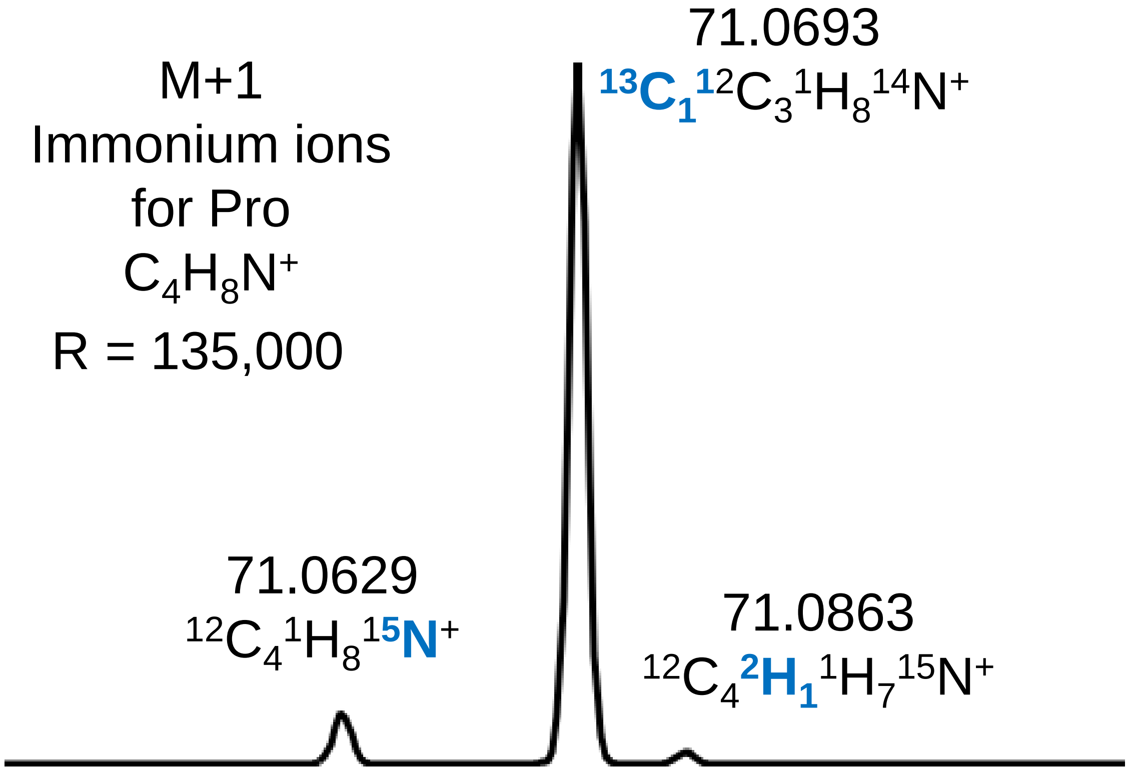
71.0863 $^{12}C_4{}^{2}H_1{}^{1}H_7{}^{15}N^+$

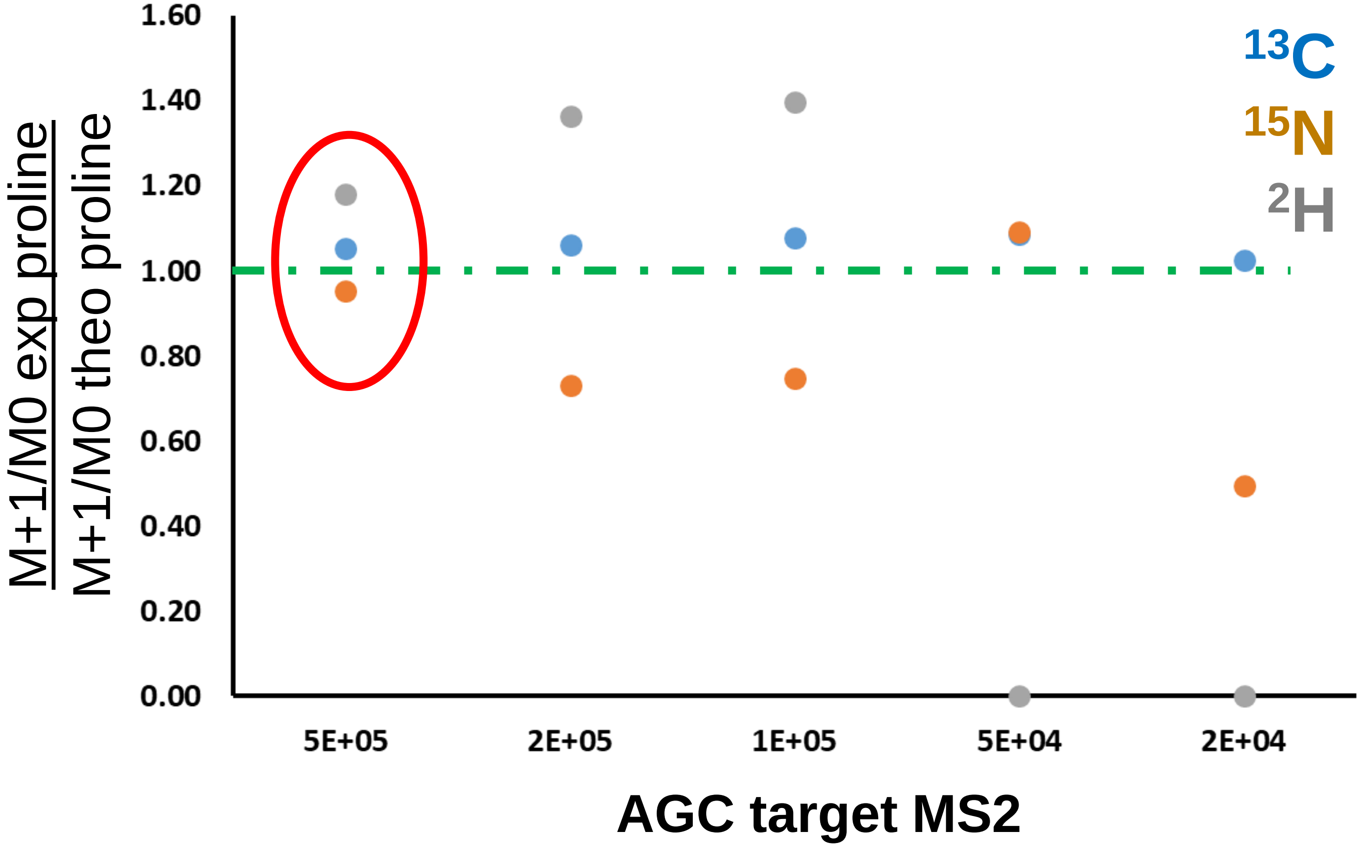
nanoLC-MS/MS
Colonne C18
MS & MS/MS **70K**
HCD 50 eV
Gradient 140 min

R = 135,000

Isotopic ratio of proline standard by direct injection on Orbitrap Q-Exactive plus

M+1
Immonium ions
for Pro
$C_4H_8N^+$
R = 135,000

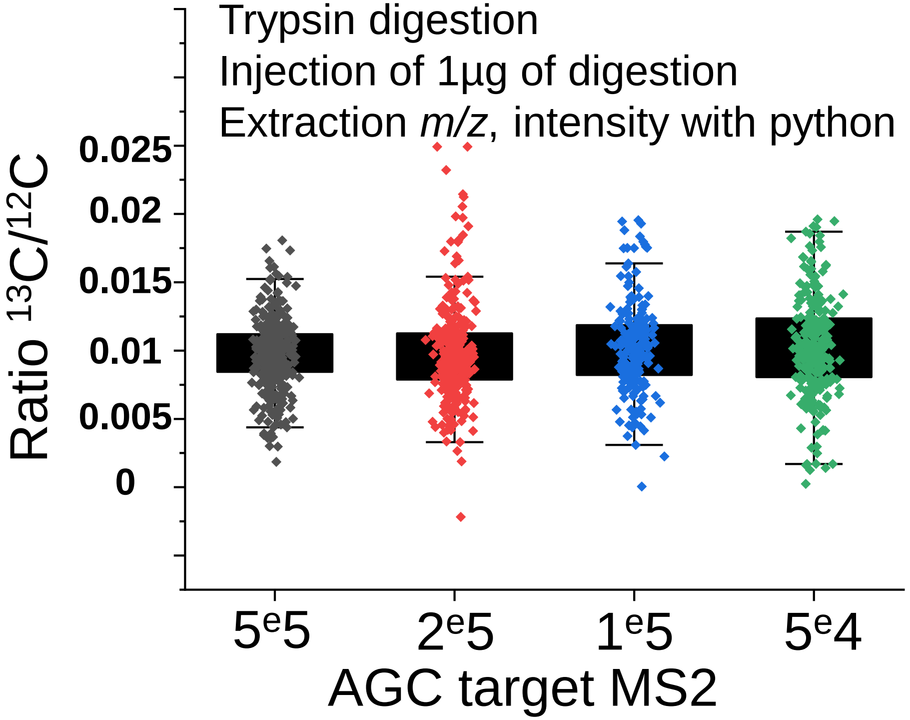


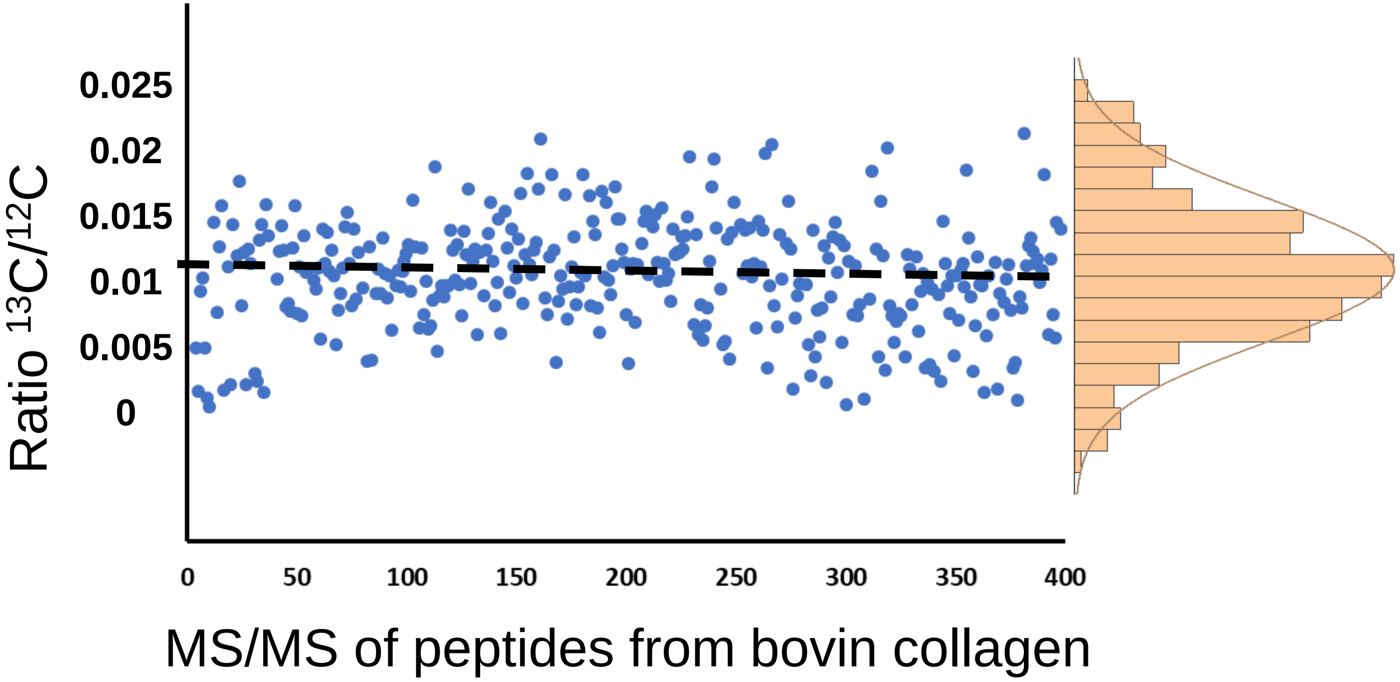


- Analysis of standard proline by infusion (1 pmol/µL) with the nanoESI Orbitrap Q-Exactive plus
- With **5e5** value, **the experimental ratio is close to theoretical ratio**
- Use of **proline to correct** data in nano LC-MS/MS

Optimization on nanoLC-MS/MS orbitrap parameters

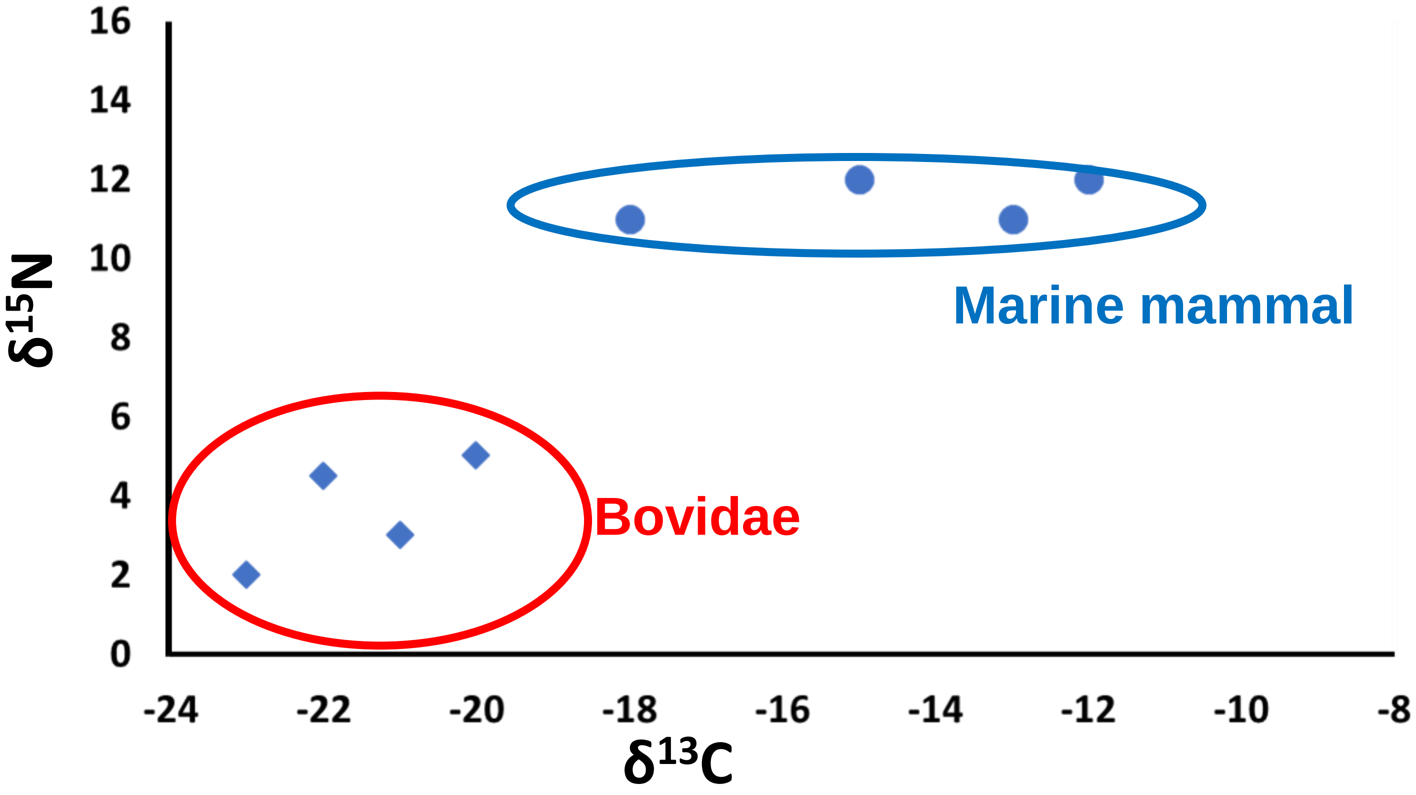
Sample: modern *Bos taurus*
Trypsin digestion
Injection of 1µg of digestion
Extraction *m/z*, intensity with python program





- **5e5 is the best AGC target** for isotopic ratio measurement on ratio **$^{13}C/^{12}C$ form proline** immonium fragment
- The distribution of isotopolog ratios is **symmetric** and the mean is 0.01058
- **High precision**, relative standard error (RSE) 1σ = **10‰**
- For **^{15}N, ^{2}H** AGC target at **5e5** is the best with low RSE

$\delta^{13}C$ and $\delta^{15}N$ for modern bones by nanoLC-MS/MS



$$\delta = \left(\frac{R\ sample/Cf}{R\ standard} - 1\right) \times 1000\ (‰)$$

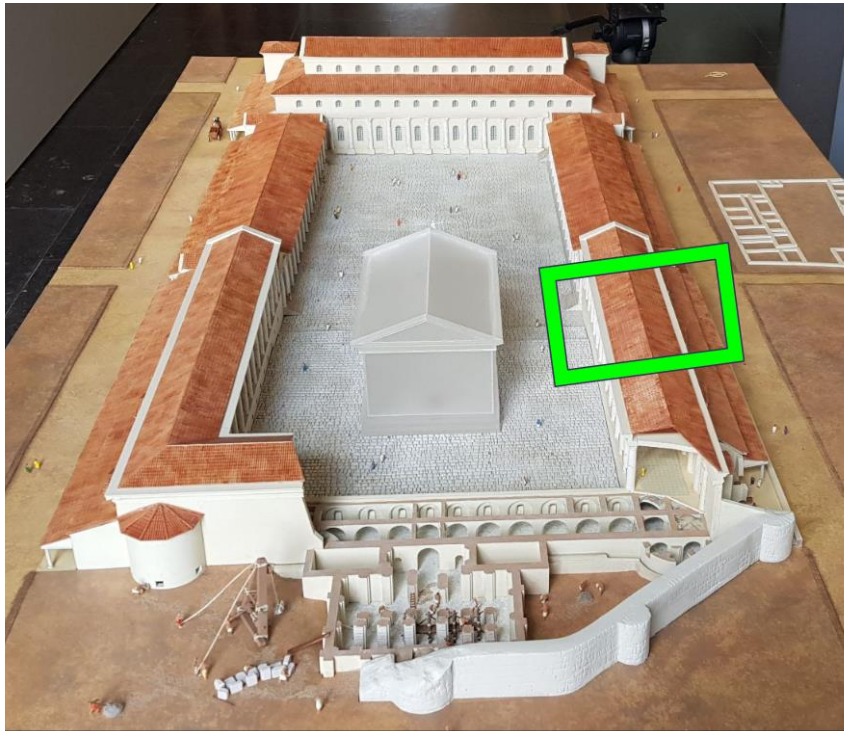
$^{13}C/^{12}C_{standard}$= 0,0112372
$^{15}N/^{14}N_{standard}$= 0,0036765
Cf = correction factor from proline

- **Isotopi**c measurements of bones by **nanoLC-MS/MS correlates** with the **bibliography**
- **Correction** of the R sample with the **R of the proline** obtained in infusion
- **Variation between samples** of the same group

Non-destructive proteomics of archaeological and paleontological bones

- Even if dating, elucidating the species and the phylogeny of an artefact or bone is a crucial point for archaeologists and collection curators, they do not want to see their **object degraded by the sampling required for analysis**.
- For museum collection, **no traces should be seen**

Unidentified bone knife handle



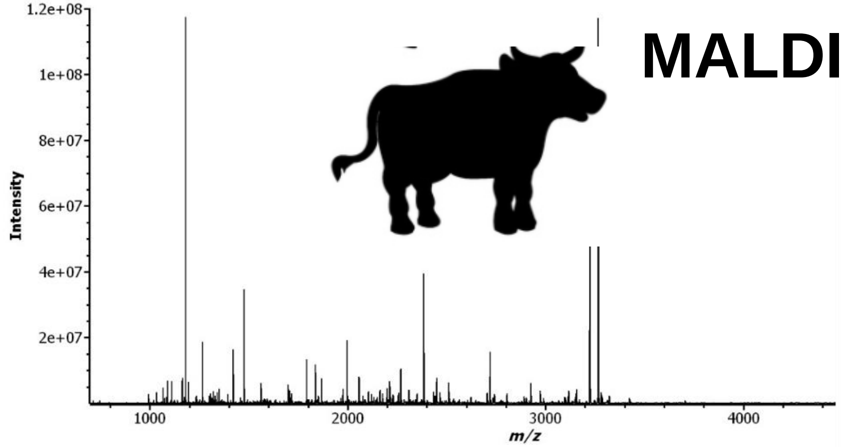
Bavay forum, Haut-de-France

Non-destructive proteomics workflow

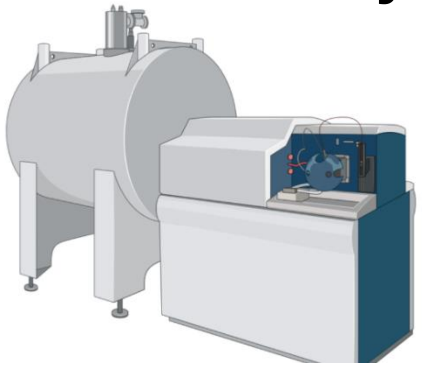
Non-destructive proteomics analysis of bones from Neolithic (10,000 BC) to Paleolithic (200,000 BC) based on **tape strip sampling**.

Neolithic bones

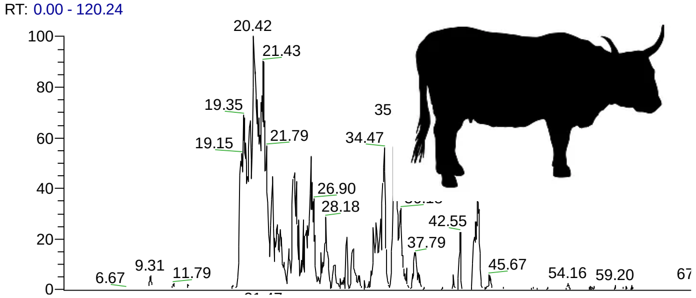
D-squame® sampling



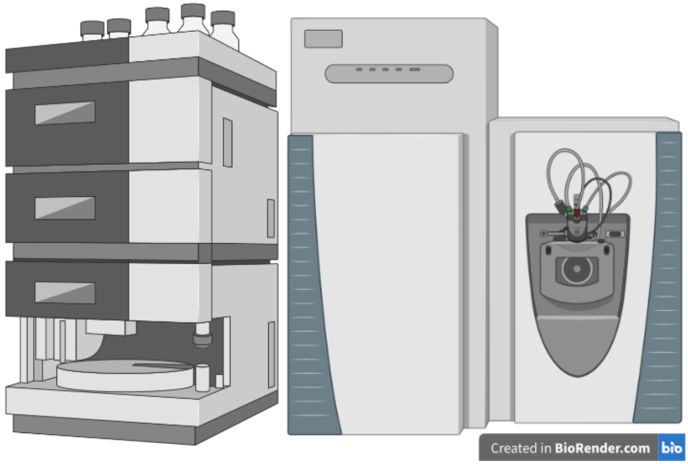
MALDI FT-ICR Analysis



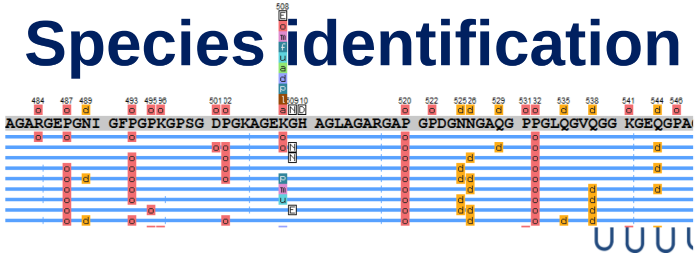
Genus identification



LC-MS/MS Analysis



Species identification

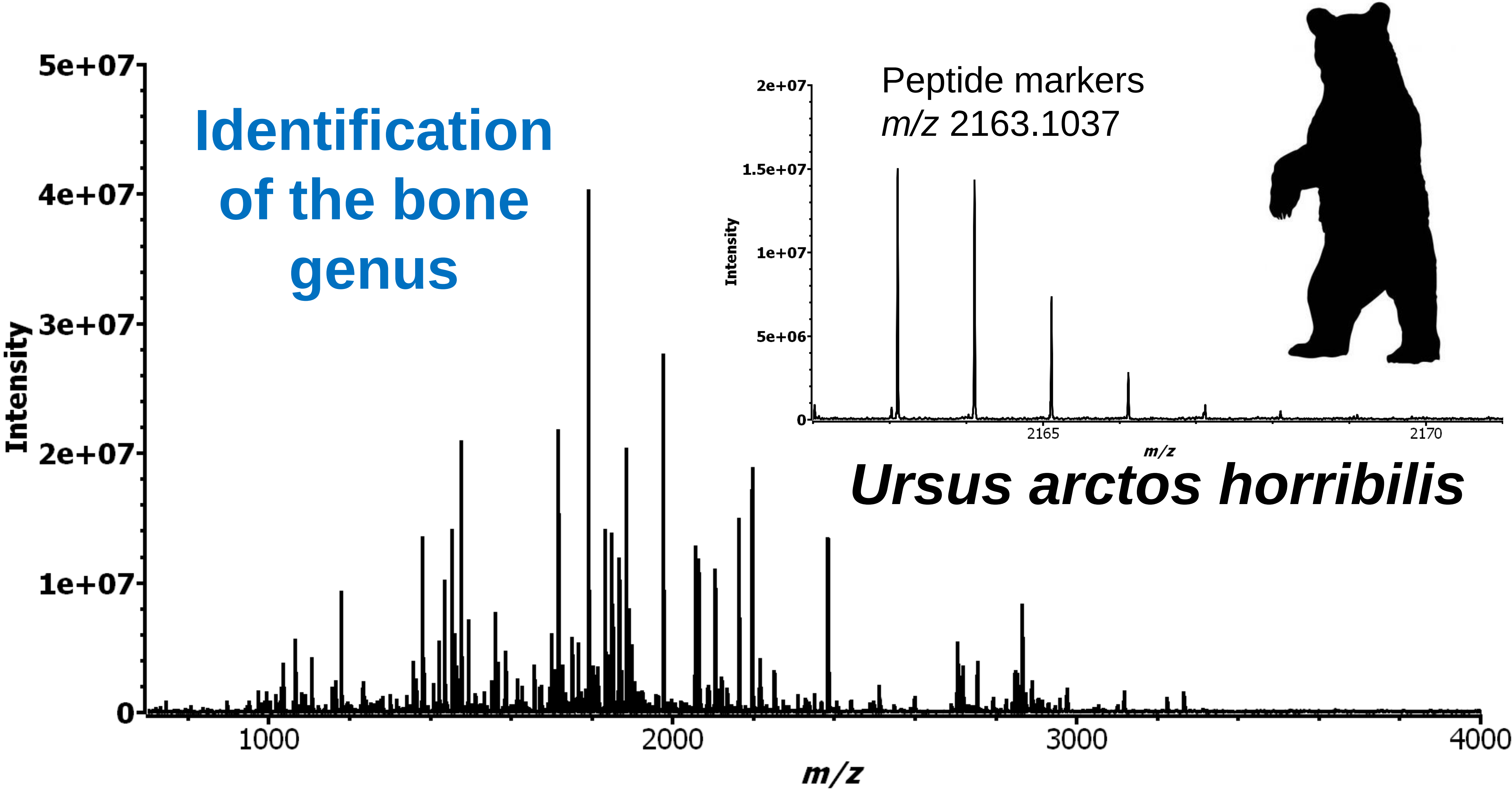


Fabrice Bray, Stéphanie Flament, Isabelle Fabrizi, Claire Delhon, Tarek Oueslati, Patrick Auguste Christian Rolando (**2023**). Non-invasive method for bone collagen identification from Neolithic until Pleistocene sample

Holocene until Pleistocene

Sample name	Site	Description	Species
Mod 1	Lille	Pelvis	*Bos taurus*
Mod 2	Lille	Femur	*Strutio camelus*
Mod 3	Lille	Pelvis	*Capra ibex*
Bou 1	Bouchain	Mandible	*Sus domesticus*
Bou 2	Bouchain	Phalanx	*Bos Taurus*
Bou 3	Bouchain	Radius	*Cervus elaphus*
Bou 4	Bouchain	Femur	*Homo sapiens*
Bou 5	Bouchain	Mandible	*Castor canadensis*
Bou 6	Bouchain	Metatarsal	*Capreolus capreolus*
Bou 7	Bouchain	Tibia	*Sus scrofa*
Bou 8	Bouchain	Femur	*Bos primigenus taurus*
Trem 1	Tremblay-en-France	Scapula	*Equus sp*
Trem 2	Tremblay-en-France	Tibia	*Bos Taurus*
Ya 1	Yanouh	Pelvis	*Ursus artos horribilis*
PE-F01	Pertus II	Radius	*Bos taurus*
PE-F21	Pertus II	Ulna	*Capra hircus*
PE-F45	Pertus II	Mandibule	*Sus sp.*
PE-F55	Pertus II	Mandibule	*Ovies aries*
PE-F72	Pertus II	Occipital	*Cervus elaphus*
Lov11	Loverval	Fragment	*Ursus spelaeus*
Wa21	Waziers	Fragment	*Equus sp*
Wa22	Waziers	Fragment	*Bos primigenus taurus*
Wa23	Waziers	Fragment	*Cervus elaphus*
Wa24	Waziers	Fragment	*Cervus elaphus ?*

Identification of species with tape strip methods by MALDI FTICR



Identification of the bone genus

Peptide markers
m/z 2163.1037

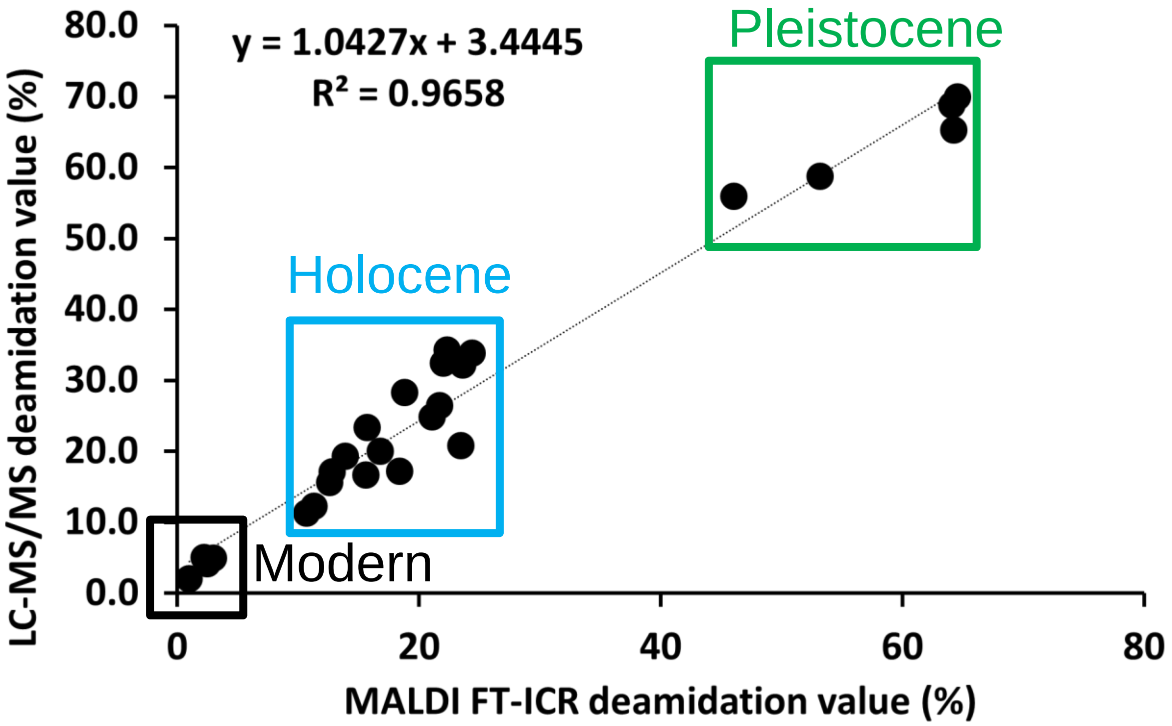
Ursus arctos horribilis

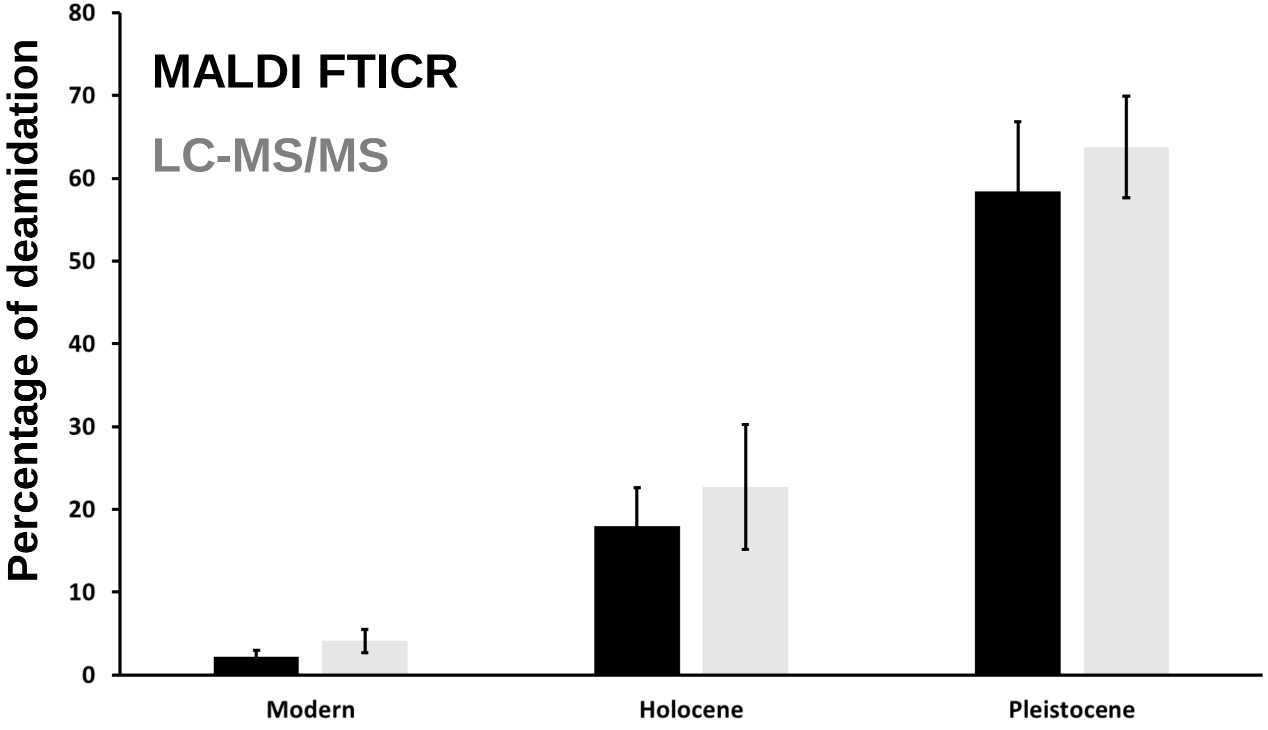
Identification of species with tape strip methods by LC-MS/MS

		1	2	3	4	5	6	7	8	9	10	11
	Species identified	***Sus scrofa***	***Bos taurus***	***Odocoileus virginianus texanus***	***Homo sapiens***	***Castor canadensis***	***Capreolus capreolus***	***Equus sp***	***Bos taurus***	***Sus scrofa***	***Bos taurus***	***Ursus arctos horribilis***
COL1A1	**Coverage**	61	55	53	52	61	60	85	58	55	61	56
	Peptides	98	132	78	135	175	81	139	233	102	111	106
COL1A2	**Coverage**	54	56	49	49	67	60	93	60	55	60	58
	Peptides	81	56	71	107	196	94	200	127	81	99	100

- The coverage of **COL1A1** varies between **52** and **85%** and of **COL1A2** between **49** and **93%**.
- The analysis shows the presence of **methylation (K), carboxylation (E), demaidation (N,Q) , hydroxylation of proline** which are present in archeological samples.

Deamidation value for modern, Holocene and Pleistocene

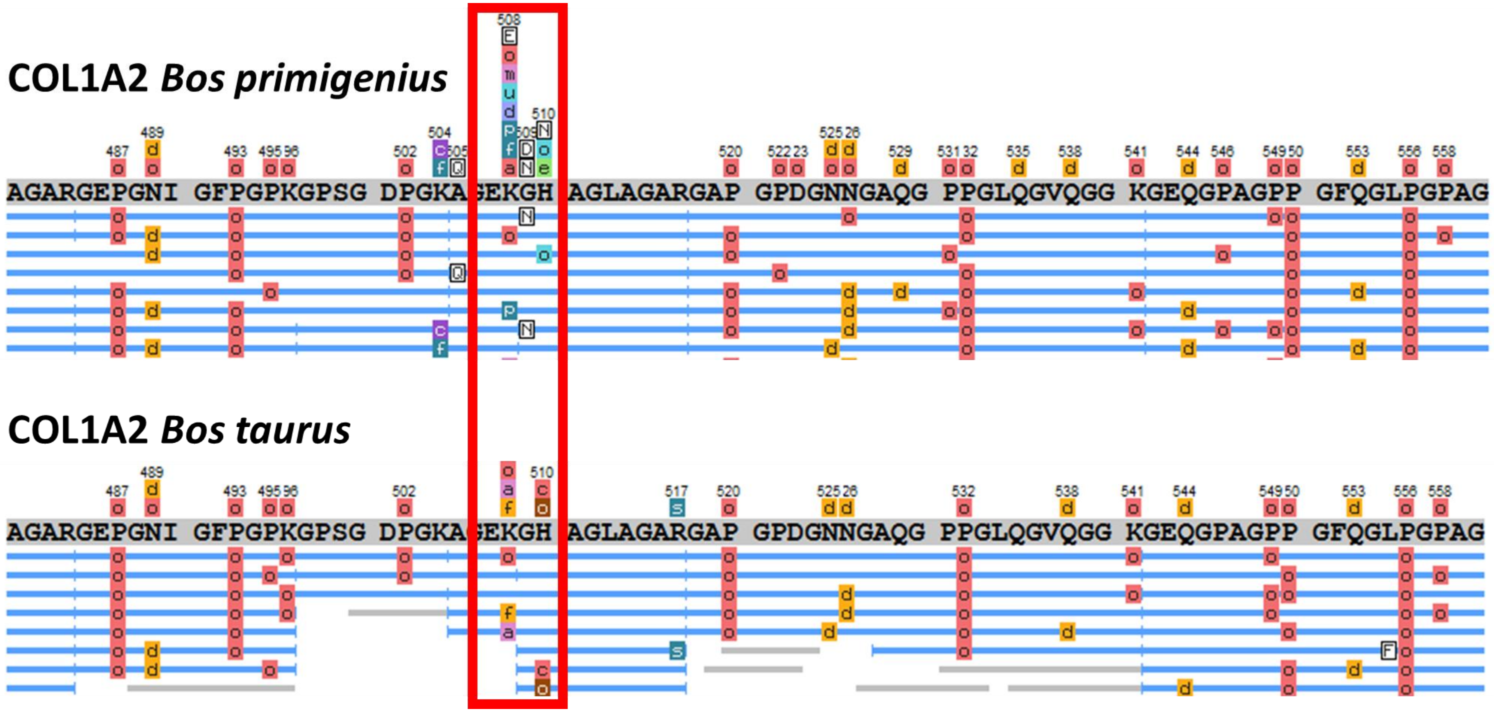




- **Correlation** of the analysis of deamidation with the 2 analytical methods **MALDI FTICR MS and LC-MS/MS**
- LC-MS/MS and MALDI FT-ICR shows a **good correlation** (**R = 0.96**)
- **Significant difference** of deamidation value

Identification of mutation on *Bos*

- LC-MSMS analysis revealed the presence of a mutation of two amino acids at positions **508 and 510** for the Aurochs sample which are not present in the samples of modern *Bos taurus.*



- These two mutations allow **to discriminate Aurochs which is the ancestor of *Bos taurus* from the modern species.**

Conclusions & perspectives

- **Optimized preparation** in 96-well plate allowing to work on 1mg of bone
- **Identification** of species of bones, micro fragments and burned bones
- **Robust quantitation of deamidation** in paleontological bones which correlates with their age
- We show for the **first time** that collagen **crosslinking is correlated with bone aging**
- **Preliminary results** on isotopic ratio measurement
- **Non destructive** methods with tape strip works on bones up to **120,000 years old**

- We are validating crosslink datation on **bones from different periods and sites**
- We are cross-correlating **datation** using **deamidation** rate and **crosslinks** quantification
- **Need decrease variation** of isotopic measurement and **correlate** data with **IRMS** experiment
- Validation of amino acid mutation **to discriminate Aurochs and Bos taurus**

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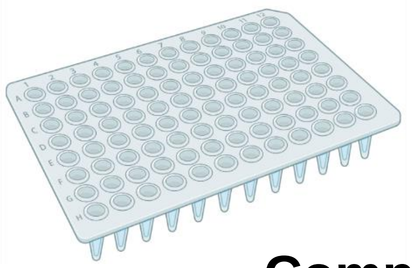
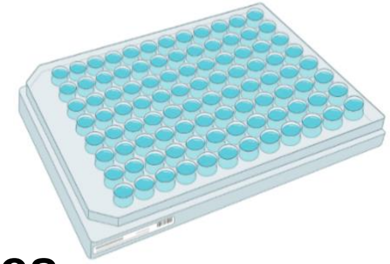
Comparison ZooMS and protein plate digestion

- **ZooMS protocol**
- Bone powder in eppendorf
- Demineralization with **HCl**
- **Centrifugation**



- **Proteins digestion on plate**
- Bone powder in filter plate
- Demineralization with **TFA**
- Filtration with **vaccum manifold**



Comparison of modern and ancient samples

- The spectra obtained for **both fractions and both methods** allows to identify bones from **1 mg** samples.

- However, the protein **plate digestion** described here allows to identify in most of the cases **most of biomarker peptides** contrary to the classical ZooMS method.

- **Similar** results with **MALDI TOF** and **MALDI FTICR**

Comparison ZooMS and protein plate digestion

			HCL 0.5 M		TFA 5%	
			Demineralized solution	**Bone powder**	**Demineralized solution**	**Bone powder**
Percentage	**MALDI FTICR**	**Modern**	15.1% +/- 8.7%	6.7% +/- 1.9%	8.6% +/- 6.3%	4.7% +/-3.4%
		Caours	65.7% +/- 8.6%	56.3% +/- 3.9%	50% +/- 4.9%	46.3% +/- 2.6%