DETERMINATION OF HYDROXYPROLINE

IN BONE COLLAGEN:

POTENTIAL APPLICATION AS A BIOMARKER FOR BONE DISEASES

BY

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Abstract

Hydroxyproline (Hyp), a non-proteinogenic amino acid is a component of the organic material in bone. It has been used for ^{14}C-dating of bone and the measurement of Hyp could be used as a biomarker in bone metabolism. Hydroxyproline is a component of collagen, the main structural protein in bone. The analyses of ^{14}C in collagen and Hyp in human bones may provide timing information about bone processes and diseases, such as osteoarthritis and osteoporosis. The analysis of Hyp in bones (e.g., the determination of Hyp content) primarily relies on a spectrometric technique, liquid chromatography-mass spectrometry (LC-MS), and the determination of ^{14}C content requires accelerator mass spectrometry (AMS). Moreover, to obtain these materials from bone requires the successful extraction of collagen and thr separation of Hyp from the collagen.

This study aims at comparing methods for extracting collagen from bone, which do not destroy the Hyp. These methods include the use of either NaOH, KOH or HCl in one stage of the extraction process and separating sufficient Hyp for ^{14}C analysis. This will provide information to determine whether Hyp can be used as a biomarker for bone diseases like osteoarthritis and osteoporosis.

A preliminary ^{14}C AMS analysis on collagen extracted by the NaOH method was carried out on human bones previously analyzed for forensic purposes. This demonstrated the ability of this technique to provide recent (post 1950) timing information.

The collagen extractions by three different methods were first conducted on modern chicken bone, and the results showed that KOH method is the best bone collagen extraction method, yielding a largest quantity of Hyp. The KOH method was then employed to extract collagen from cow bone as a test of a more human-like (mammalian) material. As this was successful, collagen was extracted from diseased human bone fragments, obtained from the Ottawa Hospital. The data revealed that Hyp was successfully obtained from these bones.

The study demonstrates that the extraction as well as the separation methods (preparative HPLC) can provide sufficient Hyp from bones for ^{14}C AMS analysis. This will lead to future studies of Hyp in bone turnover, which may lead to its use as a novel biomarker for bone diseases such as osteoarthritis and osteoporosis.

Acknowledgements

I would like to express deep gratitude to my past supervisor Dr. Jack Cornette who passed away in October 2017. Jack was very supportive throughout this project. His enthusiastic attitude toward to medical projects, such as disease related to bone, inspired me to continue this project. Also, I would deeply thank Dr. Liam Kieser who supported me working on my project by contacting the radiocarbon lab, the department of biology, and the Ottawa Hospital which helped me to finish my project. Really. I appreciate that he spent time with me and gave me the chance to complete my dream to get a master's degree here in Canada. I would like to specially acknowledge the efforts of radiocarbon lab staff (Sarah Murseil, Carley Crann, Carolyn Dziwa) and biology department (Linda Kimpe, Ammar Saleem) who taught me the ropes in the lab and they have been a mentor to me over this year and we've had a great time working together, learning, debating, and talking science. Also, thanks so much for team from the Ottawa Hospital (Dr. Mark Campbell, Katherine Reilly) who provided the sample of human bone to find the goal of my project. I was also lucky to work with a great team who are the most caring and dedicated advisors I have ever met. Thanks for Barbara Francisco who help me to organize my materials and all the equipment that I need to work in Lab. I would like to thank my parents for their unconditional love and support and for never letting me forget that family comes first. Also, I will not forget my country Saudi Arabia that gave me the scholarship to study in Canada, really thanks so much for giving this chance. Finally, I would like to thank my brother who came with me to live in Canada and for keeping me fueled with delicious curries, motivating me every day to keep on track, and for always making sure I am smiling.

Table of Contents

List of Abbreviations

AMS	= Accelerator mass spectrometry
^{14}C	= Carbon-14 or radiocarbon
HCl	= Hydrochloric acid
Hyp	= Hydroxyproline
KOH	= Potassium hydroxide
LC-MS	= Liquid chromatography – mass spectroscopy
NaOH	= Sodium hydroxide

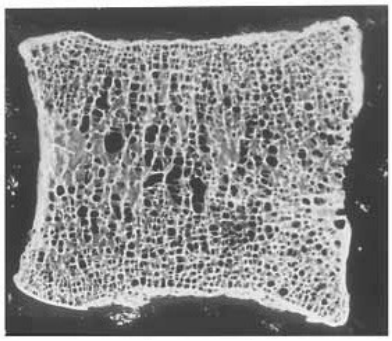
Chapter 1

Introduction

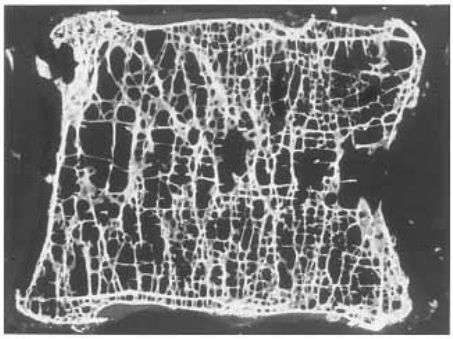
Bone is partly composed of organic material (30%), versus 70 % in most soft tissue. Collagen is about 90% of the organic material. Thereby it is different from other types of tissues in the body tissue (Young, 2003). It is commonly accepted that collagen plays critical roles in the structure and function of bone tissue. Collagens consist of a large family of multimeric proteins with up to 38 genes, coding 20 different collagens. The major collagen triplex in mineralized tissues is type-I and is composed of two α1 chains (also called COL1A1 or α1[I]) and one α2 chain (COL2A1 or α2[I]). Mutations that lead to defects in type-I collagen assembly can also cause Osteogenesis Imperfecta (‘‘brittle bone’’) (Primorac et al., 2001).

Osteoporosis occurs when bone resorption is more active than bone formation. It is characterized by low bone mass and microstructural deterioration of bone tissue (Fig. 1, top), which result in increased fragility and susceptibility to fracture risk (Wheater et al., 2013).

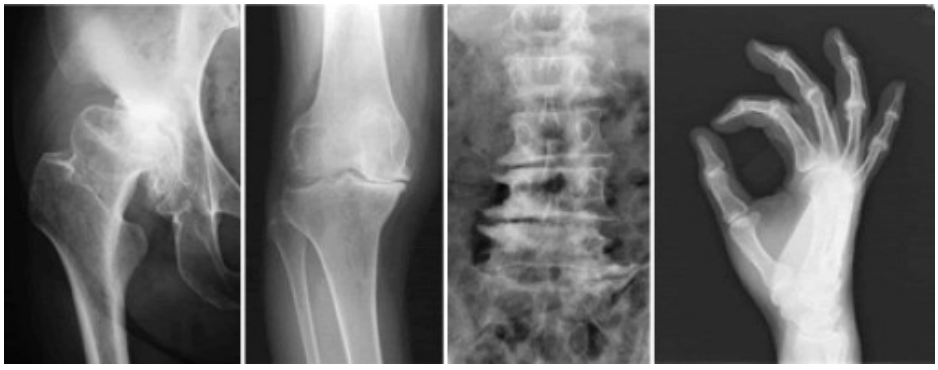
On the other hand, osteoarthritis (or OA), which can occur at all joints of the body, is characterized by two aspects: (i) cartilage degradation in joint space narrowing observed in radiography; and (ii) osteophyte formation at the edge of the joints (Kawaguchi, 2016) (Fig. 1, bottom).



Matrix of Normal Bone



Matrix of Osteoporotic Bone



Hip Knee Lumbar Hand

Figure 1 TOP: The matrix of normal bone and osteoporotic bone. The matrix of normal bone is dense, whereas that of osteoporotic bone is porous (Image was adopted from *Kovacevic, 2017)*. **BOTTOM**: Radiographs of osteoarthritis in hip, knee, lumbar spine, and hands. Two major disorders of osteoarthritis are cartilage degradation in joint space narrowing and osteophyte formation at the edge of the joints. (Image was adopted from Kawaguchi, 2016).

Hydroxyproline is a major component of collagen (accounted for roughly 13.5% of mammalian collagen) and plays a key role in maintaining collagen stability by permitting the sharp twist of collagen helix. Thus, the analysis or diagnosis of bone, which can indicate diseases such as osteoporosis, can rely on the presence of Hyp in bone collagen. Hydroxyproline, abbreviated as Hyp, was first isolated from hydrolyzed gelatin by Hermann Emil Fischer in 1902 (Fischer, 1902). The naturally occurring Hyp is in L stereoisomer, so called L-hydroxyproline or L-Hyp. It is a common non-proteinogenic amino acid that differs from proline by the presence of a hydroxyl (OH) group in the gamma carbon atom. L-hydroxyproline has a chemical formula of $C_5H_9O_3N$, and its chemical structure is illustrated in Fig. 2.

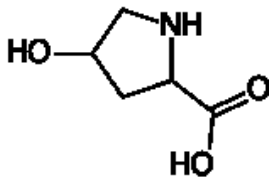


Figure 2 Chemical structure of L-hydroxyproline (L-Hyp).

Hydroxyproline has been used for dating of bone owing to the presence of the radioisotope of carbon, carbon-14 or ^{14}C, as one of its atomic components (Stanford et al., 1982). ^{14}C is continuously formed by the interaction of cosmic rays with nitrogen atoms in the upper atmosphere where it combines with oxygen for form CO_2. It enters plant tissue through photosynthesis and is acquired by humans from through the ingestion of animals and plants. Since its discovery by Willard Libby in 1946 (Libby, 1946), the ^{14}C-dating method has been widely developed and applied to

various fields, such as in the study of metabolic activities of plants, animals, and human (Cook and Mackenzie, 2014).

^{14}C is not only used for dating old material; it can also be used for recent events (post 1950) with good precision and this help to understand processes in human bone.

Moreover, the ^{14}C-dating method has also been employed to study and understand different body parts in various aspects. This can include the study different parts and types of bone, such as collagen and osteon, cortical and trabecular bone, and other parts, to understand bone turnover as well as proteins causing bone diseases such as osteoporosis (Cook et al., 2015).

The ^{14}C-dating method has also been largely utilized to study human bone. It has recently been applied for forensic purposes (Cook and Mackenzie, 2014), in which the calibration for the time since 1950 when nuclear testing in the atmosphere occurred ("bomb peak") is used. For example, in the study of a biological profile of human remains by analyzing the ^{14}C content in bone and tissue (Cook et al., 2015). In addition, using a different calibration, it has become a standard tool in archaeological study, for example, the content of ^{14}C were compared with a calibration curve to investigate the age of a mummy found in the western part of Pakistan (Kretschmer et al., 2004): by comparing the ^{14}C content with a calibration curve (Reimer et al., 2013), the calibrated year that the mummy died could be determined.

Although ^{14}C activity of human bone collagen significantly lags behind the activity in a range of organs and soft tissues, the study of its ^{14}C content can be beneficial in understanding the bone collagen turnover. According to the bone turnover study by

Hedges et al., 2007, the ^{14}C content was analyzed in adult human femoral mid-shafts, and they found that the bone turnover of individuals between the ages of 20 and 80 years is ≤4 %. They found that during adolescent growth of 10 - 15 years of age, the collagen turnover is higher at 5 to 15% per year. There is, however, a significant decrease in collagen turnover to 1.5% per year after the age of 19, which is considered a termination of puberty. These have provided additional perspective on the impact of the aging process on bone collagen turnover. Moreover, studies of bone Ostional turnover by Shin et al., 2004, indicated that the fraction of bone remodeled per year defines a turnover rate, which is known to vary between skeletal elements, depending on the applied chronic stresses and age. They estimated the rate of cortical bone turnover to be in the range of 2 - 8% per year. As we learn more about the biochemical properties of bone matrix proteins such as collagen, it is possible that new markers could be developed as indicators of osteoporosis and osteoarthritis. *In fact, previous studies used urine or blood hyp samples which evaluate whole-body Hyp (*Kuo and Chen Biomarker Research 2017). The level of Hyp was shown to significantly increase in urine with postmenopausal women with osteoporosis, in comparison with the postmenopausal healthy women. The increase of urinary Hyp indicates that the degradation of collagen type-I from the bone matrix is raised in osteoporotic women. Hyp is derived from newly synthesized procollagen peptides during bone formation. Moreover, Hyp can be found in other tissues such as skin and cartilage and also can be liberated from the metabolism of elastin and C1Q. Consequently, detecting urine or blood Hyp is considered as a non-specific method. Thus, in my research, determination of Hyp in collagen directly from the bones of osteoporotic patients is a specific and direct method to identify when the Hyp was produced and so help to determine its efficacy as a biomarker for Bone

Resorption which is linked to Osteoporosis. Hyp provides about 12–14% of the total amino acid content of mature collagen. During the degradation of bone collagen, about 90% of the HYP is released and then the Hyp is primarily metabolized in the liver.

Thus, this study has two primary goals:

(1) To compare Hyp-containing bone collagen extraction methods (NaOH, KOH, and HCl methods).

(2) To establish a preliminary Hyp-based ^{14}C-dating of bone, which can be used as a potential biomarker for osteoporosis.

The experiments were conducted by first comparing three extraction methods, including NaOH, KOH, and HCl methods, which were used to extract collagen from chicken bone. The extracted Hyp was subsequently subjected to analysis by liquid chromatography-mass spectroscopy (LC-MS). The best method, which resulted in highest yield of Hyp, was then applied to extract collagen from cow and human bones. In addition, elemental analysis (EA) of collagen extracted from chicken bone was also carried out.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Bone samples

Three types of bones (chicken, cow, and human bones) were used in this study. All bones contained lipid and fat upon received. The description of each bone sample, including type of bone, sex, age, body part, bone provider, preservation method, and temperature, are tabulated in Table 1. Examples of human hip bones provided by patients with end-stage OA who were undergoing total hip replacement (THR) are shown in Fig. 3. These human bone samples were accessible during the course of the data collection, which was approved by the Ottawa Health Science Network Research Ethics Board.

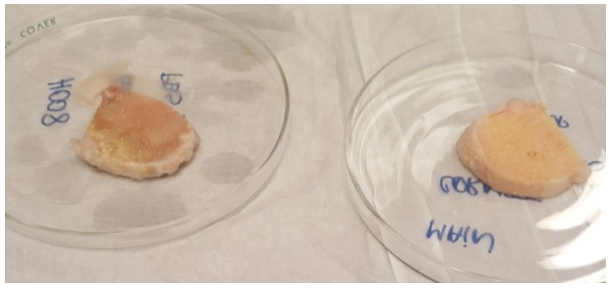


Figure 3 Photograph of examples of human hip bone samples from patient with end-stage OA taken for THR, showing that there was a significant loss of cartilage due to OA.

Table 1 Bone samples used in this study.

Type of bone	Sex	Age (years old)	Body part	Provider	Preservation method	Preservation temperature
Chicken	Female	Adult, modern	Leg	Ottawa market	Refrigerated	4 ^{o}C
Cow	Female	Adult, modern	Leg	Butcher market	Refrigerated	4 ^{o}C
Human (H007)	Male	73	Hip	Ottawa general hospital	Refrigerated in PBS buffer	4 ^{o}C
Human (H008)	Female	70	Hip	Ottawa general hospital	Refrigerated in PBS buffer	4 ^{o}C
Human (H009)	Male	65	Hip	Ottawa general hospital	Refrigerated in PBS buffer	4 ^{o}C
Human (H010)	Female	70	Hip	Ottawa general hospital	Refrigerated in PBS buffer	4 ^{o}C

2.1.2 Chemicals and instruments

Chemicals and instruments other than those described in methods section are listed in Tables A1 and A2, respectively, in the appendix.

2.2 Methods

2.2.1 Extraction of bone collagen

A diagram showing the overall process of collagen extraction is shown in Fig. 4. This process was established for the ^{14}C dating of archaeological bone material which had often been in contact with soil for long periods of time. Therefore, the process begins with an acid wash (HCl, 0.5N) to remove any accreted carbonates which could alter the ^{14}C content. This is followed by a base wash (0.1N) to remove any humic acids which might have accreted onto the bone. In principle, these steps should not be necessary for modern bone which has not been buried; however, when comparing the hyp yields from the collagen during this work, it was found that the use of a base, specifically KOH was necessary to obtain the Hyp. It is suspected that the base in some way dis-entangles the collagen fibers so that the Hyp is more readily released.

Following the base wash, HCl is added to bring the pH to 3 and the sample is left overnight to gelatinize. The gelatin is then freeze dried and forms a white to yellow powder.

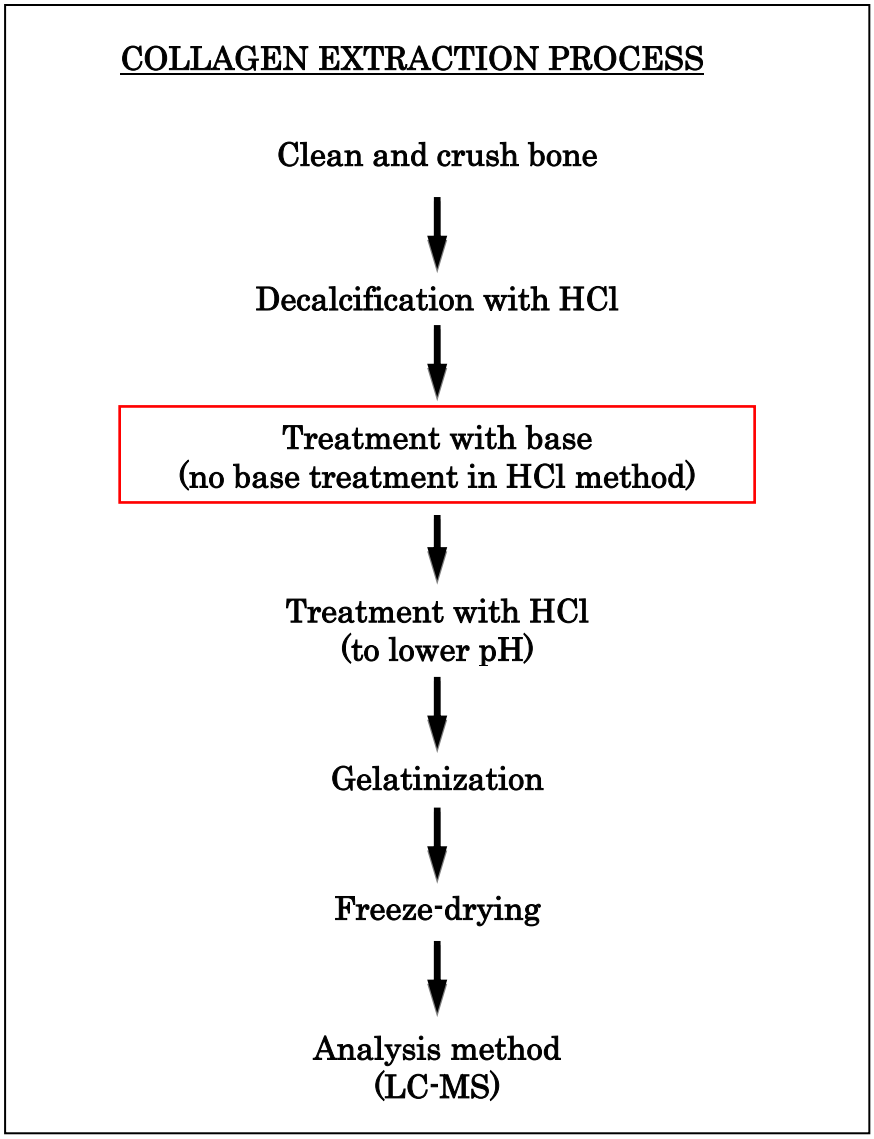


Figure 4 Diagram showing the overall collagen extraction process. Step in the red box is varied among the three methods.

As shown in Table 4, the extractions of collagen from chicken bones were carried out using KOH, NaOH, and HCl methods, whereas those from cow and human bones were carried out using only KOH method.

Table 4 Types of bones and extraction methods.

Type of bone	Extraction method
Chicken	NaOH, KOH, and HCl
Cow	KOH only
Human	KOH only

To find the most efficient collagen extraction method, three different extraction methods (KOH, NaOH, and HCl) were compared. The general procedure in extracting collagen was performed following to the well-established methods (Longin 1971; Brock et al., 2007; Waters, 2015).

In a typical process, bones that were scraped clean of contaminants, were wrapped in an aluminum foil and then dried in an oven. After that, the bones were crushed into cm-size pieces prior to being ground in a coffee grinder machine. One gram of ground bone was weighed into a Pyrex® tube, and subsequently subjected to decalcification in 0.5 N HCl (Fisher Scientific) for 18 h (HCl was changed 3 times) at room temperature (20 ºC). Prior to gelatinization, the decalcified bone was alternately treated with 0.5 N HCl and rinsed with MilliQ® water at room temperature until the solution's pH was decreased to 3.0. The gelatinization was then conducted in HCl, pH 3 at 75ºC for 20 h, followed by centrifugation. Finally, the samples were

freeze-dried and then subjected to analytical methods. The differences of each method are as follows:

2.2.1.1 NaOH method

After decalcification, bones were treated with 0.1 N NaOH (Fisher Scientific) for 30 min at room temperature (Longin 1971; Brock et al., 2007).

2.2.1.2 KOH method

The extraction of collagen using KOH method was based on the well-established method by Water, 2015. In this method, the decalcified bones were treated with 0.1 N KOH (Fisher Scientific) for 2 - 3 days at 4 ^{o}C.

2.2.1.3 HCl method

In this method, bones were not treated with base (KOH nor NaOH), but were directly subjected to gelatinization.

2.2.2 Analysis methods

Three different methods, including high performance liquid chromatography (HPLC) (Fig. 5), liquid chromatography-mass spectroscopy (LC-MS), and accelerator mass spectroscopy (AMS), were employed in this study.

In the determination of Hyp content, LC-MS was employed. Prior to sample injection, the freeze-dried collagen samples were hydrolyzed and then mixed with 1 mL of a solution containing water and 0.1% formic acid. Some operating conditions of the methods are summarized in Table 5.

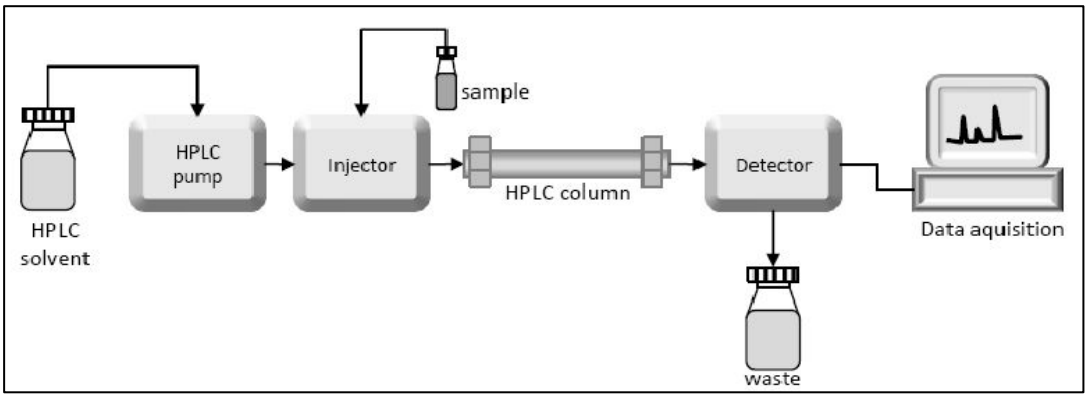


Figure 5 Process diagram for HPLC.

2.2.2.1 LC-MS analysis

Prior to LC-MS analysis, dried collagen samples were dissolved in Omnitrace grade water (Fisher), vortexed and filtered to 0.2 µm. The analysis was performed on a Shimadzu HPLC coupled to a Sciex 4000 Linear Ion Trap Quadrupole MS/MS. Samples were injected at a volume of 1 µl onto a 60ºC-heated Acquit UPLC BEH-C18 1.7 µm, 2.1 x 100 mm column using an isocratic flow of Omnitrace water at 0.2 ml/min and a total run time of 5 min. The mass spectrometer was operated in positive electrospray ionization mode with source temperature of 500 ºC, and decluttering and entrance potential of 61 and 10, respectively. Masses of 132.062 with 86.000, and 132.062 with 68.000, were monitored using collision energy of 19 and 31, respectively, and collision cell exit potential of 14 and 12, respectively.

Table 5 Summary of some operation conditions for LC-MS.

Analysis method	Operating conditions
LC-MS	• **Mobile phase 1:** Water and 0.1 M formic acid • **Mobile phase 2:** Acetonitrile and 0.1 M formic acid • **Column:** UPCL BEH-C18 2.1 x 100 mm; particle size, 1.7 micron

2.2.2.2 Accelerator mass spectrometry (AMS) analysis

^{14}C occurs in the atmosphere today at a level of one atom in 10^{12} ^{12}C atoms. AMS is the only technique that is sensitive enough for such measurements. It achieves this sensitivity in 4 ways:

a. It can produce large currents of negative ions from the sample which provide sufficient numbers of the rare ^{14}C ions to provide a statistically significant result.
b. By selecting negative ions, atoms of other elements that have the same mass (isobars), ^{14}N in the case of ^{14}C, are eliminated (^{14}N cannot make negarive ions)
c. By changing from negative to positive ions in the accelerator, sufficient electrons are removed from the ion that molecules with the same mass (molecular isobars) are destroyed.
d. The positive ions emerge from the accelerator with sufficient energy (4% of the speed of light) that the detector can count them one at a time with no noise.

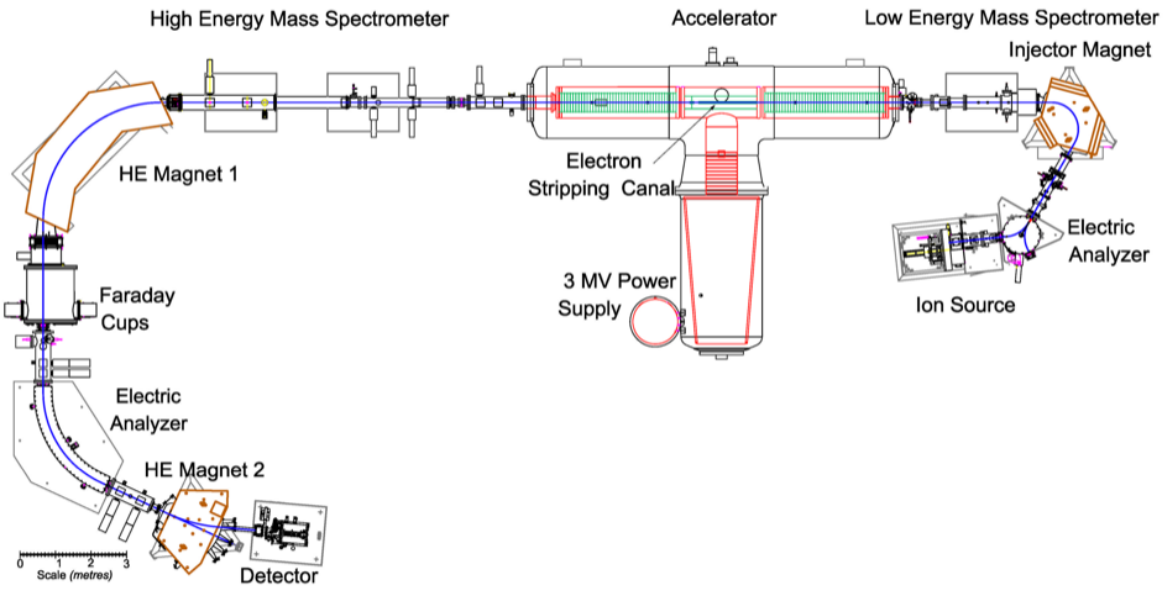


Fig 6. Schematic diagram of the AMS system. The blue line shows the ion beam path.

The amount of carbon obtained from Hyp, extracted by the LC-MS is not sufficient for AMS analysis. However, as a test of the procedure, a preliminary AMS experiment was conducted in forensic human bones. After collagen was extracted by NaOH method, the following procedures, including combustion and graphitization of collagen, and analysis of ^{14}C by AMS, were carried out.

2.2.2.2.1 Combustion and graphitization of collagen

Collagen was weighed into tin capsules and then subjected to EA combustion to CO_2 using a Thermo Flash 1112 elemental analyzer (EA) in CN (carbon, nitrogen) mode interfaced with a manual extraction line to trap the pure CO_2 in a pre-baked 6 mm Pyrex breakseal. Blank tin capsules were also combusted between each sample to monitor the blank and to ensure no memory effect. Samples of pure CO_2 in 6 mm breakseals were converted to elemental carbon in the presence of iron and hydrogen using semi-automated graphitization lines, which were designed and built in the Lalonde AEL-AMS Laboratory (St-Jean et al., 2017).

In brief, the graphitization reaction ($CO_2 + 2H_2 \rightarrow C_{(s)} + 2H_20_{(s)}$) takes place at 550 °C. Over 2-3 hours, elemental, solid carbon was produced on 5 mg of preconditioned (oxidized and reduced) -200 mesh iron powder and solidH_2O was removed cryogenically at -40 °C. The pressure, oven temperature, and cooling bar cup temperatures for each sample were monitored and graphed until no further changes in pressure were observed and the reaction was complete. The graphitized samples were then pressed into aluminum targets with copper press-pins using a pneumatic press, designed and constructed in-house.

2.2.2.2.2 Analysis of ^{14}C by AMS

The measurement of ^{14}C was performed on a 3 MV tandem accelerator mass spectrometer built by High Voltage Engineering (Kieser et al. 2015). C^{+3} ions were measured at 2.5 MV terminal voltage with Ar stripping. The fraction modern carbon, $F^{14}C$, was calculated according to Reimer et al., 2004 as the ratio of the sample $^{14}C/^{12}C$ ratio to the standard $^{14}C/^{12}C$ ratio (Ox-II) measured in the same data block. Both $^{14}C/^{12}C$ ratios were background-corrected to a machine blank and the result was corrected for natural, spectrometer, and preparation fractionation using the AMS-measured $^{13}C/^{12}C$ ratio normalized to $\delta^{13}C$ (PDB). ^{14}C ages were calculated as $-8033\ln(F^{14}C)$. The errors on ^{14}C ages (1σ) were based on counting statistics and $^{14}C/^{12}C$ and $^{13}C/^{12}C$ variation between data blocks.

Chapter 3

Results and Discussion

3.1 Comparison of Hyp in collagen extracted from chicken and cow leg bones by different extraction methods

Prior to extracting collagen from cow and human bones, the preliminary extraction was carried out on chicken leg bone obtained from the Ottawa market. Chicken leg bone was chosen not only because it can be easily obtained from a local market, but it also contains high Hyp content. In general, the content of Hyp in collagen content is approximately 13.5% (Kliment et al., 2011). In other words, in every 100 g of collagen, there is 13.5 g of Hyp.

Figure 7 Photograph of examples of freeze-dried collagen extracted from chicken bones. Collagen extracted by NaOH or HCl method is white, whereas that extracted by KOH method is yellow.

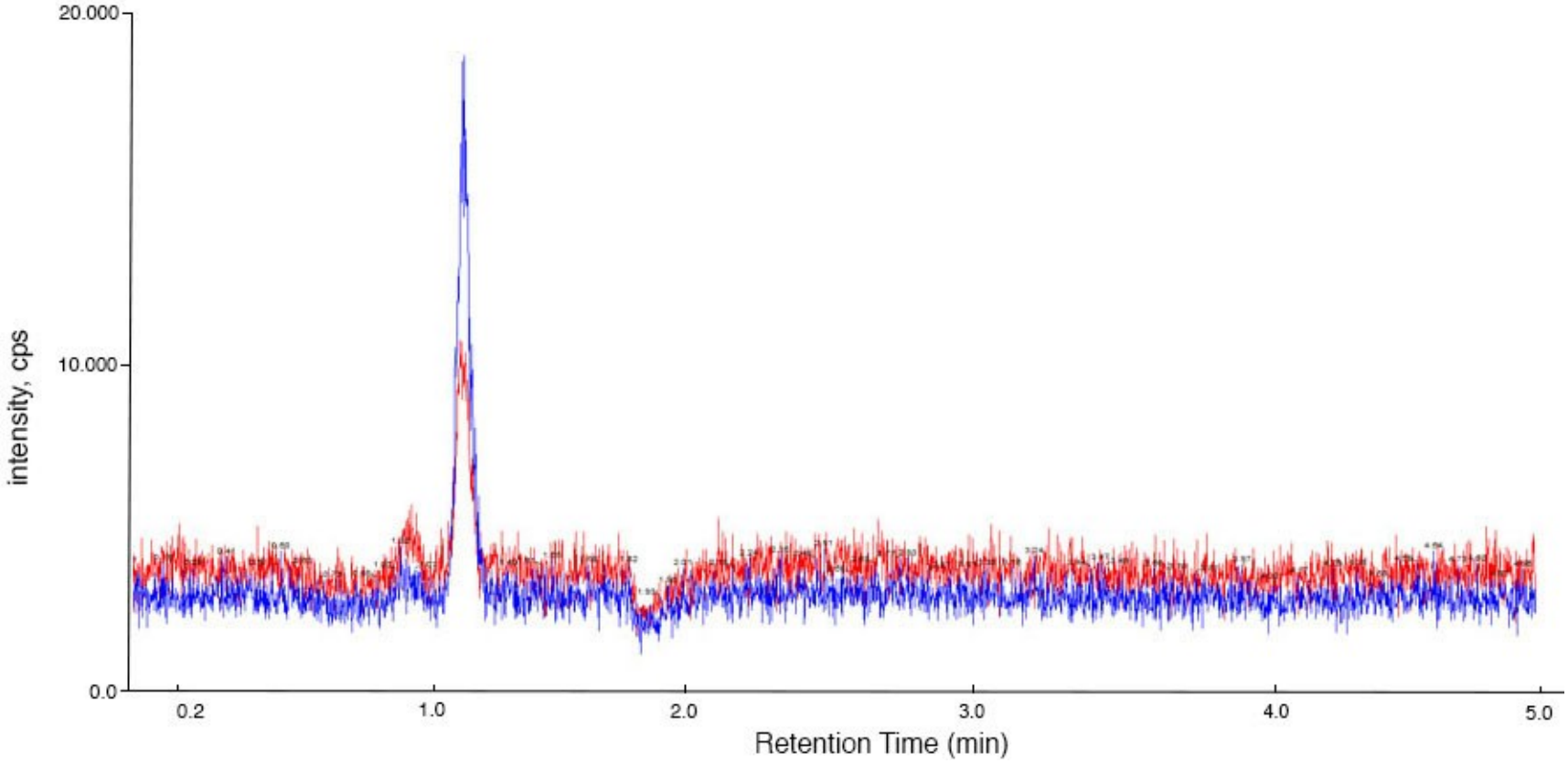


Figure 8 Example of LC profile for standard Hyp.

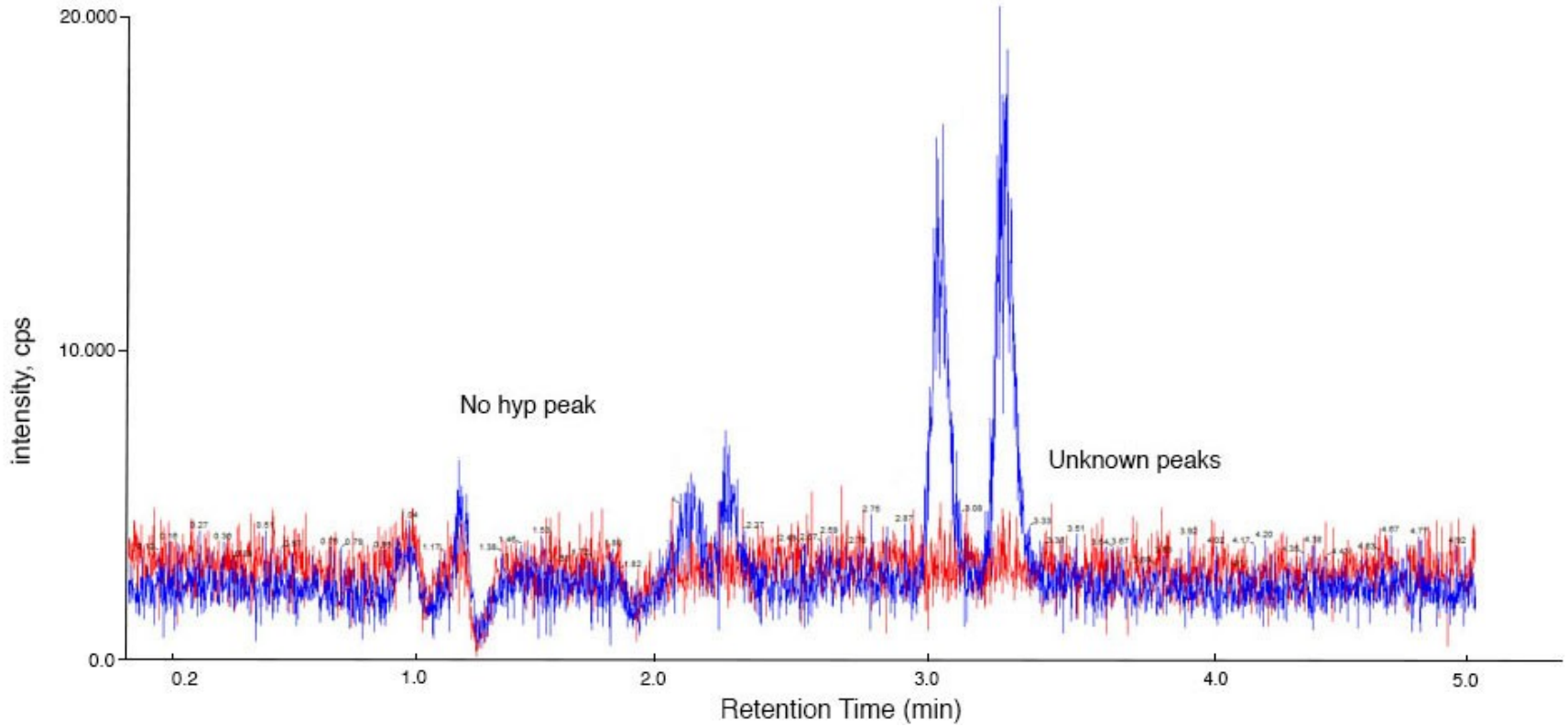


Figure 9 Example of LC profile for Hyp in collagen extracted from chicken bone by NaOH

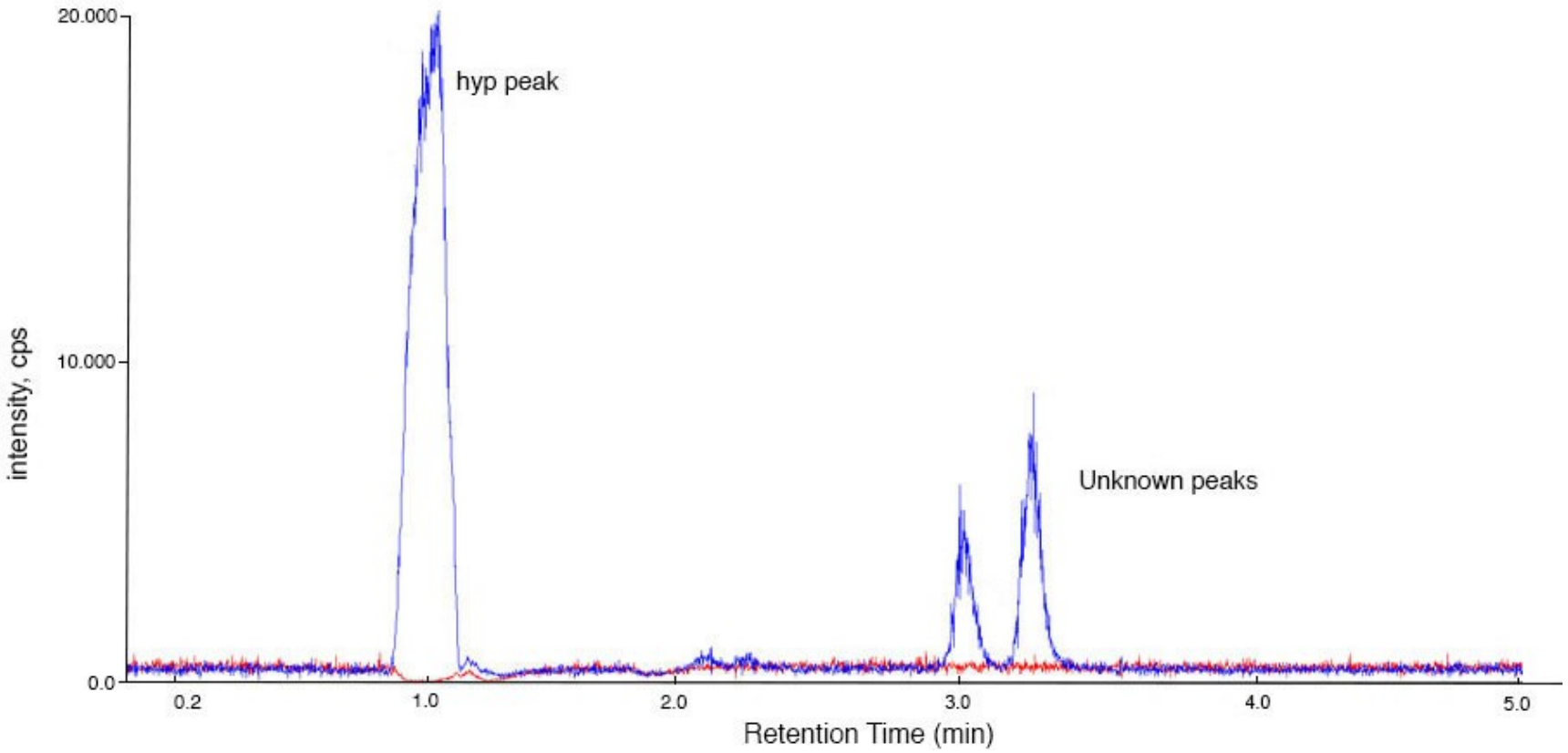


Figure 10 Example of LC profile for Hyp in collagen extracted from chicken bone by KOH method.

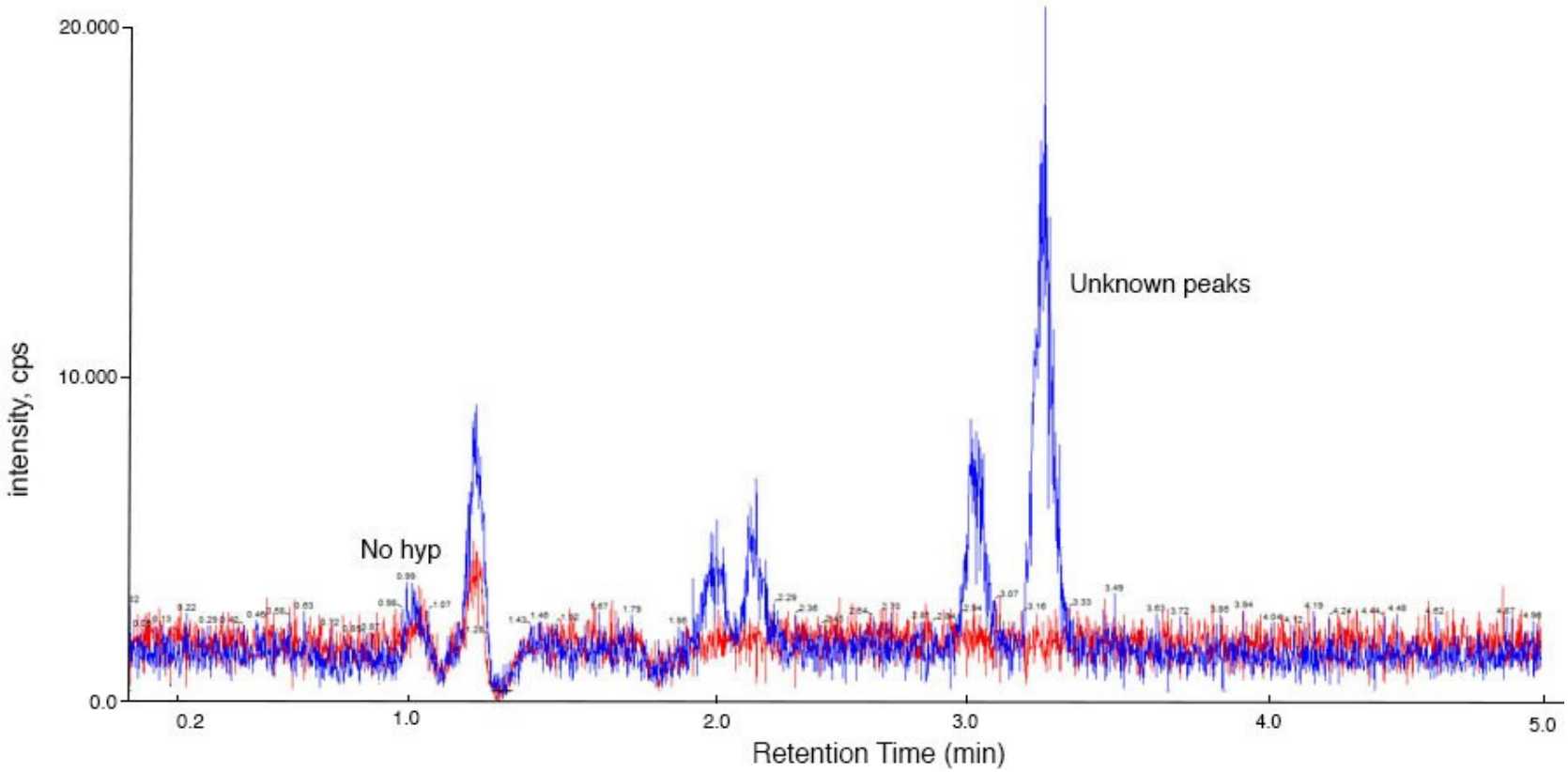


Figure 11 Example of LC profile for Hyp in collagen extracted from chicken bone by HCl method.

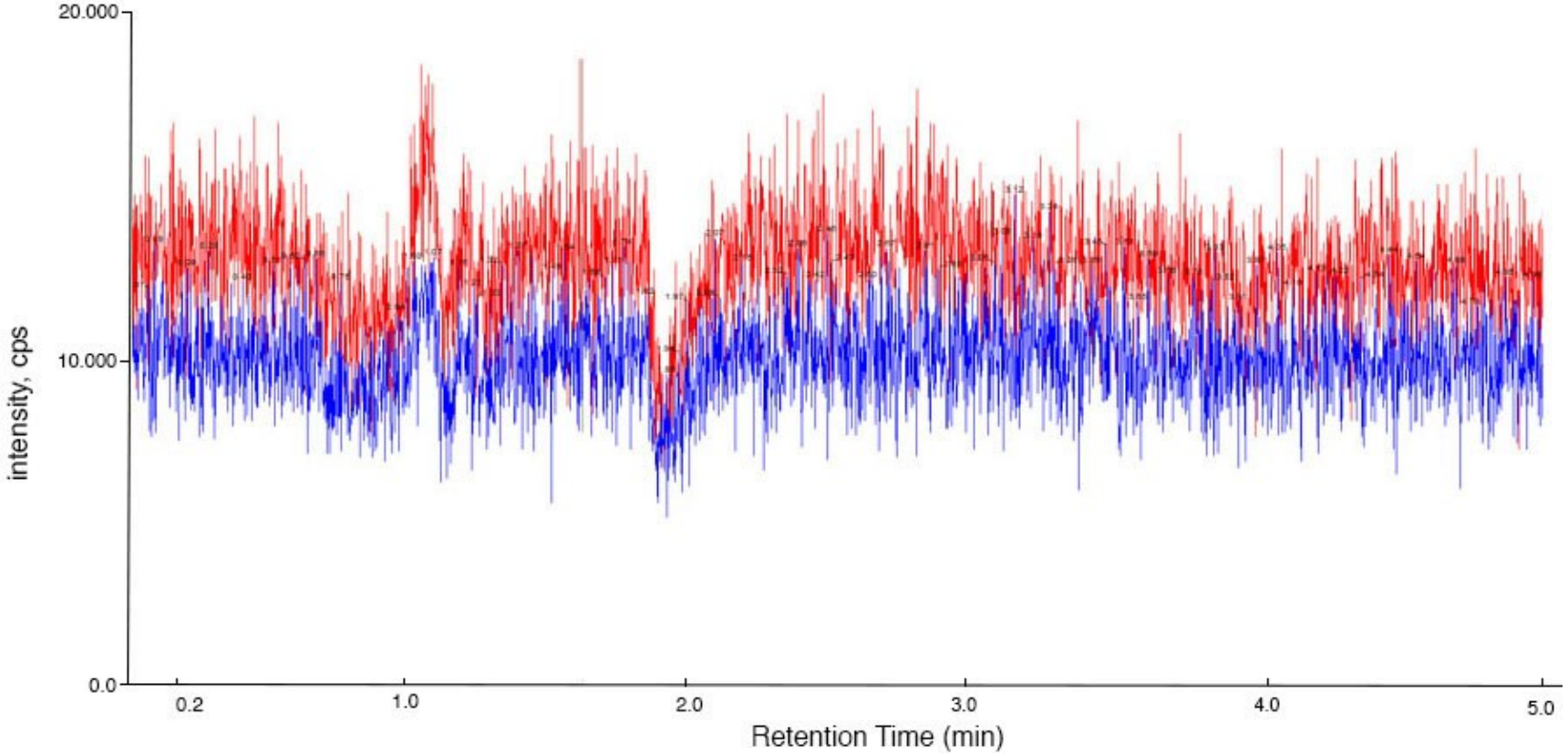


Figure 12 LC profile for blank control (water).

According to the literatures, Collagen is extracted from bone mainly by two methods: NaOH method (Longin 1971; Brock et al., 2010) and KOH method (Waters et al., 2015). In these methods, bones are treated following decalcification with a basic solution of NaOH or KOH to extract collagen. However, in this study, the extraction without basic solution (so called HCl method) was also attempted.

In the final step of the extraction method, collagen was freeze-dried to obtain collagen powder. Interestingly, the color of freeze-dried collagen extracted by NaOH and HCl method is white, whereas that extracted by KOH method is yellow (Fig. 5). This indicates collagen extracted by KOH method may contain some residual chemicals. The obtained collagen was further hydrolyzed prior to analysis by LC-MS. Figures 8-12 show the examples of LC profile of Hyp in collagen extracted from chicken bone collagen using different extraction methods as well as those of the Hyp standard and blank control (Fig. 12). The LC profile of the Hyp standard indicates a single distinct peak (Fig. 8). The peak of Hyp obtained by KOH extraction method is highest (Fig. 9), compared with those obtained by NaOH (Fig. 9) and HCl (Fig. 11) methods. This demonstrates that KOH is a better Hyp extraction method.

To confirm the identity of Hyp observed in LC peak, the LC-purified Hyp extracted with NaOH and KOH was further subjected to MS analysis. Compared with those of the LC-purified Hyp extracted with NaOH method (Fig. 14 and blank control (Fig. 15 the MS profile of the LC-purified Hyp extracted with KOH method showed a distinct peak corresponding to Hyp at m/z = 132.15 ([M+H]+1) (Fig. 13). These data further confirmed that the KOH method is a better Hyp extraction method; thus, was subsequently used for extracting Hyp from cow bone.

The quantification of collagen and Hyp extracted from chicken and cow bones by different extraction methods are summarized in Table 6. The data indicate that the KOH method was successful applied to extract Hyp from cow bones. Nonetheless, the expected ^{14}C concentration obtained from cow bone did not meet the target level; therefore, is not suitable for AMS analysis. It is likely that larger size of samples is needed to achieve the expected ^{14}C concentration that meet the target level.

In contrasts to the results reported by Nalawade-chavan et al., 2014, in which NaOH and HCl were compared, demonstrating that strong base has better performance in extracting collagen from old bones, the peak of Hyp from modern bone collagen extracted by HCl method (Fig. 11) is higher than that extracted with NaOH method (Fig. 9). This may suggest that the extraction of collagen from modern bone can be more effective without base treatment; however, further studies should be conducted. These preliminary results clearly indicate that KOH method is the best collagen extraction method, which results in highest yield of Hyp, and thus of expected carbon.

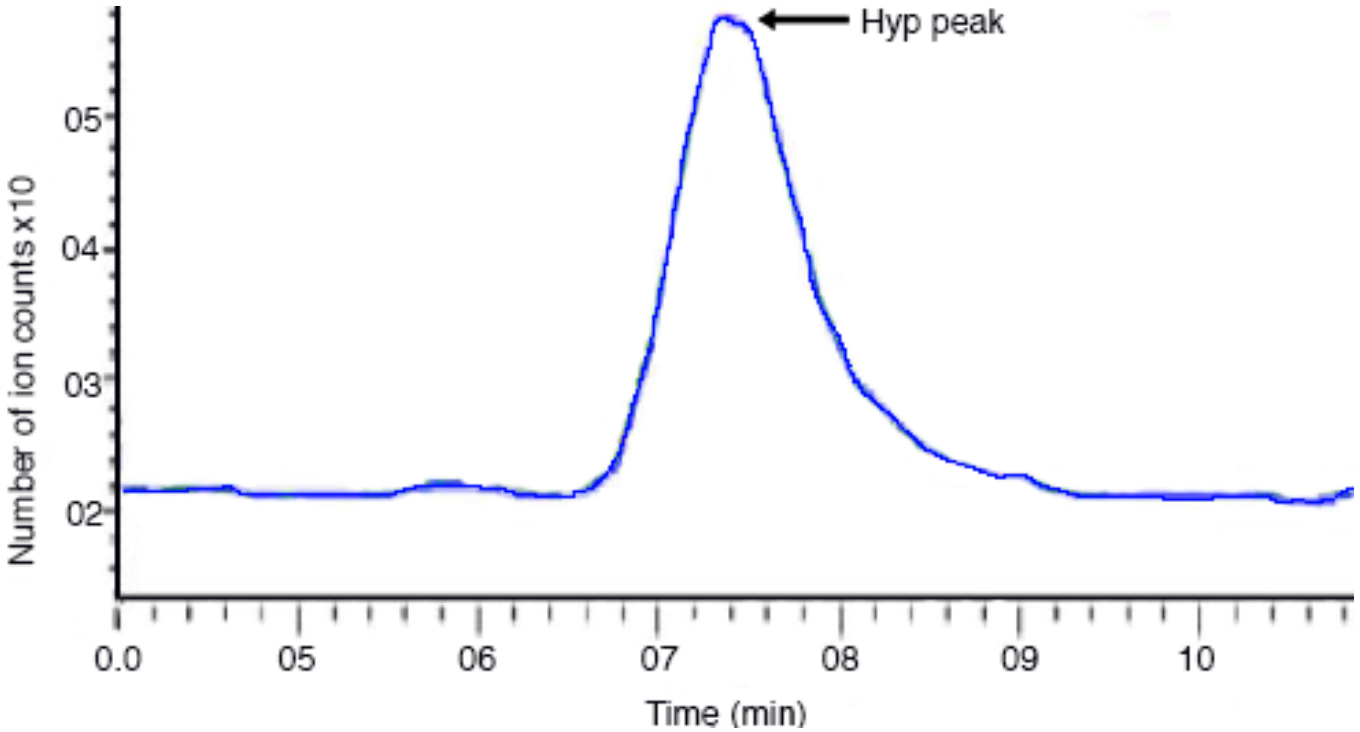


Figure 13 Example of an MS profile (number of ions of a particular mass counted as a function of time) for Hyp in collagen extracted from chicken bone by KOH method. The blue line shows the number of molecules with mass 132.15 amu.

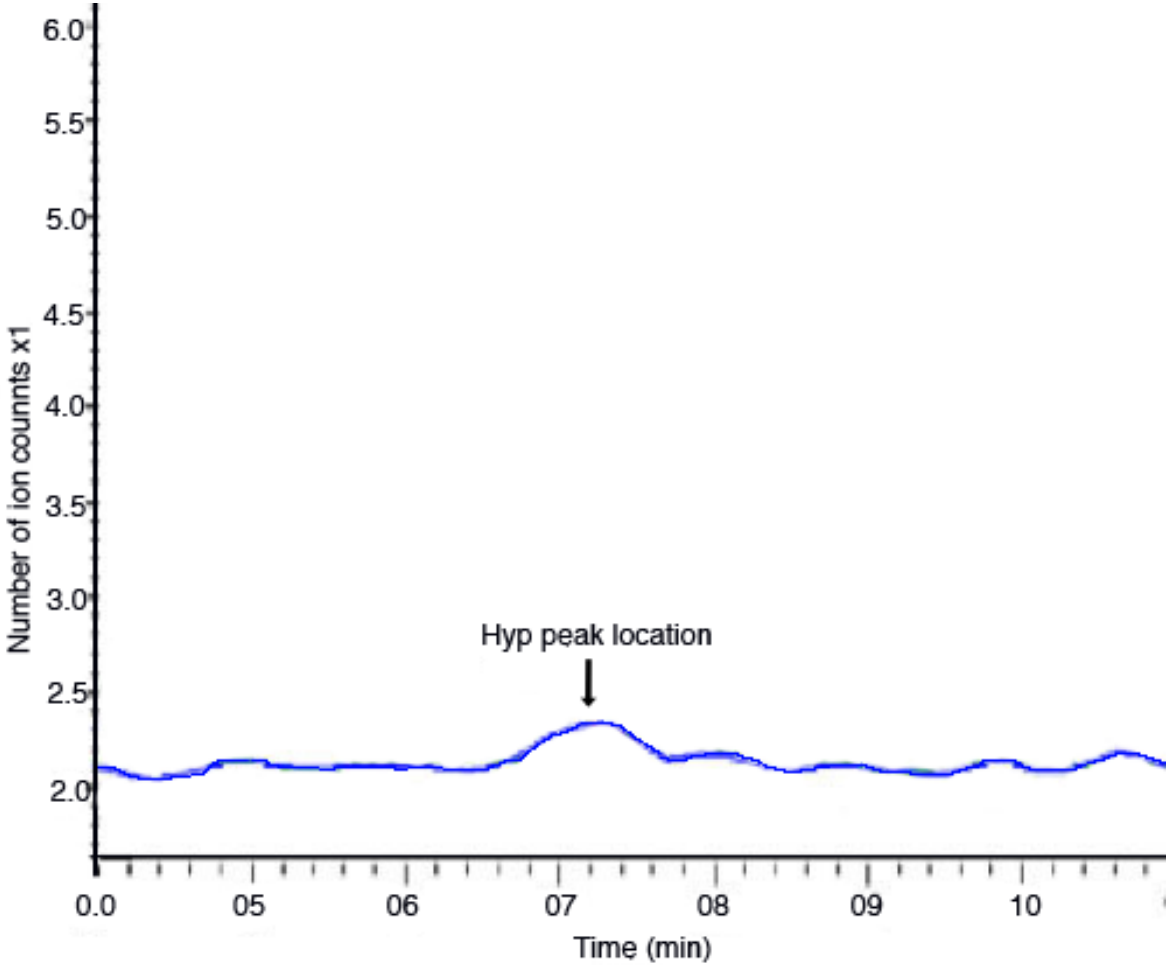


Figure 14 Example of MS profile for Hyp in collagen extracted from chicken bone by NaOH method. See Fig 13 caption for details.

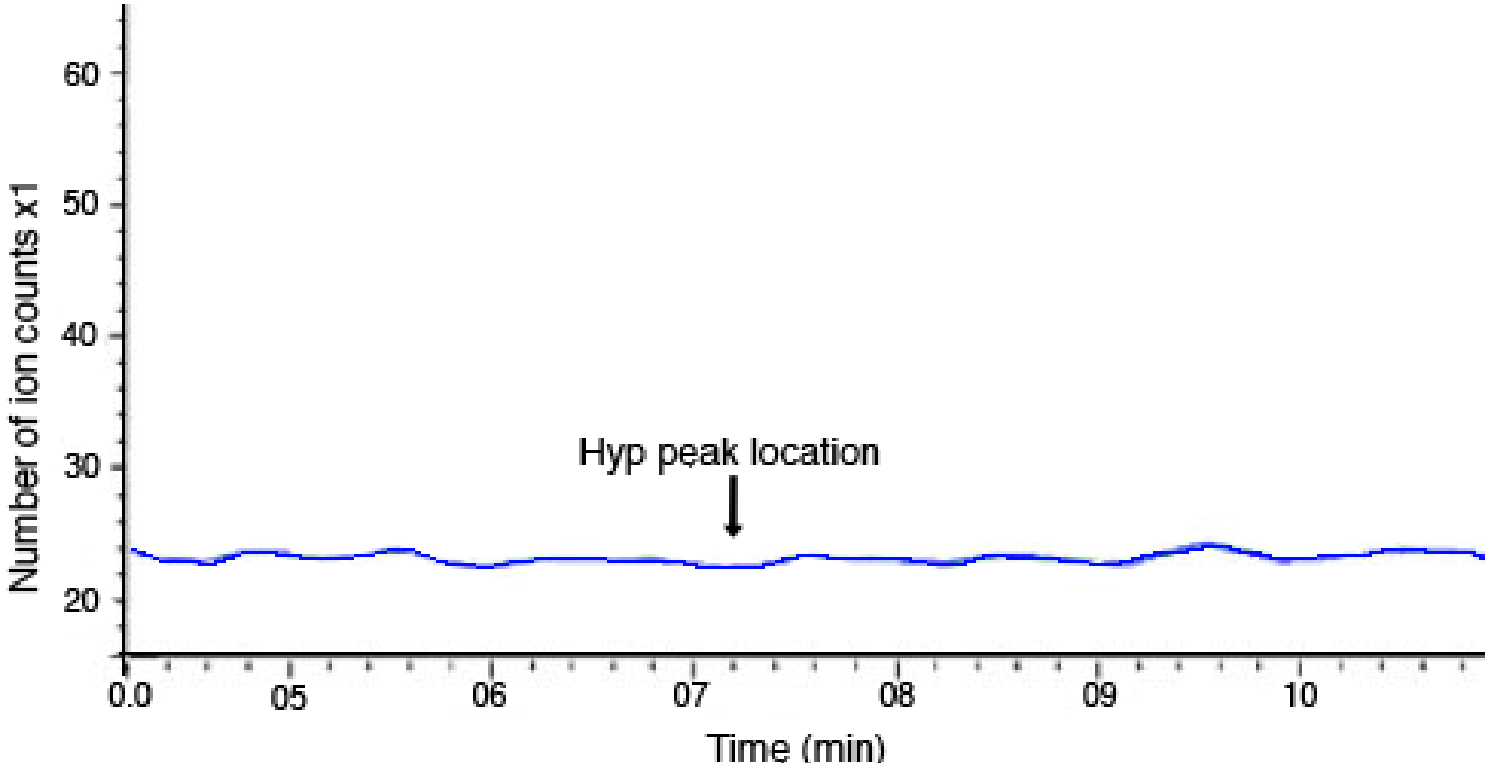


Figure 15 MS profile for blank control (water).

Table 6 Contents of Hyp and collagen extracted from chicken and cow bones by different extraction methods.

Type of bone	Extraction method	Weight of dried bone (g)	Weight of dried collagen (mg) in LCMS	Hyp content (μg/g collagen)	Expected carbon content (μg/g collagen)
Chicken*	NaOH	0.070	20.65	0.00	0.00
	KOH	1.037	26.52	**1466.8**	671.8
	HCl	6.934	31.34	133.06	5.58
Cow**	NaOH	N.D.	N.D.	N.D.	N.D.
	KOH	80	4600	**1039.3**	475.98
	HCl	N.D.	N.D.	N.D.	N.D.

NOTE: N.D. = Not determined.
*Lipid was not removed from bone prior to collagen extraction.
**Lipid was removed from bone prior to collagen extraction.

3.2 EA data of collagen extracted from chicken bone by KOH method

Collagen extracted from chicken bone by NaOH and KOH method was further compared and quantified by EA. The results are tabulated in Table 7. Surprisingly, the carbon content in collagen obtained from NaOH was 1.07, where that obtained from KOH method was 0.76, indicating that the collagen obtained by KOH method may contain some residual chemicals, as also indicated by its yellow color. Although this amount of carbon would be sufficient for AMS analysis, in this case the analysis was not done.

Table 7 EA data showing content of carbon in collagen extracted from chicken bone by NaOH and KOH methods

Extraction method	Mass of collagen (mg)	% Nitrogen	% Carbon	C:N ratio	Carbon content (mg)
NaOH	2.343	15.88	45.46	2.45	1.07
KOH	2.521	4.94	30.28	5.25	0.76

3.3 Determination of Hyp in collagen extracted from human femoral head bones by KOH method.

Based on the preliminary data, which indicated that collagen can be successful extracted by KOH method, the method was further applied to extract collagen of human bones. Collagen was extracted from four human femoral bone samples, namely H007 – H010, and the results are summarized in Table 8.

The data showed that the extraction of collagen by KOH method was successfully applied to human hip bones, despite the fact that no Hyp was detected in two (out of four) human bones, possibly due to that the amount of Hyp in these human hip bones are under the detectable threshold. This suggests that more advanced instrument, such as preparative HPLC, may be needed to analyze hyp at such trace concentration.

Table 8 Contents of collagen and Hyp from human hip bones extracted by KOH method and analyzed by HPLC.

Bone	Weight of dried bone (g)	Weight of dried collagen* (mg)	Hyp content in LCMS peak (µg)	Carbon content in LCMS peak (µg)
H007	3.83	28.00	0.00	0.00
H008	3.29	30.89	0.675	0.309
H009	3.74	29.42	0.095	0.044
H010	3.13	29.65	0.000	0.000

Note: Lipid was removed from bone prior to collagen extraction.

*Weight of dried collagen used for LC-MS analysis

Table 9 Comparison of the demographics of sources of human hip bones with Hyp content.

BMD is Bone Mineral Density; T-score is a T-score -0.1 or above is normal density bone but the low of that means Osteopenia.

Bone	Sex	Age (years old)	Time from diagnosis to surgery (month)	Hip BMD (g/cm^2)	T-score	Hyp content in LCMS peak (μg)	Carbon content in LCMS peak (μg)
H007	Male	73	18	N.D.	N.D.	0.00	0.00
H008	Female	70	**9**	0.936	-0.6	0.675	0.309
H009	Male	65	**8**	1.119	0.7	0.095	0.044
H010	Female	70	36	1.055	0.4	0.000	0.000

These bones were from femoral heads of patients who have been diagnosed with femoral head bone osteoarthritis. These patients have different demographics, in terms of sex and age, as well as different bone density, the relationship of such factors with respect to the content of Hyp was thus further analyzed and compared. The low number of samples, required that a non-parametric statistical analysis be used. Linear regression was used to evaluate the association between demographic factors (Table 9) and Hyp. Spearman correlation coefficients were calculated for which a p-value of <0.05 was considered statistically significant, were performed using SPSS 16.0 (SPSS, Chicago, IL). The data are tabulated in Table 9. A preliminary analysis on the demographics and Hyp data (from Human 1 and 4) can be found in Tables A.8 and A.9 in the Appendix).

Although the highest yield of Hyp occurred in a patient with a relatively good BMD, there does not appear to be a strong correlation between the two measurements. The two highest Hyp values also corresponded to the two patients with the shortest time from diagnosis of hip osteoarthritis to surgery. This could indicate that those with higher bone turnover may be at higher risk for osteoarthritic progression, suggesting that the determination of Hyp in bone may be used as a novel biomarker for osteoarthritis. The current method for evaluating bone density through a **"**Bone Scan" called "Bone Densitometry. The gold standard in bone mass measurement and fracture prediction is dual energy X-ray absorptiometry (DEXA or DXA), used primarily for the hip and spine. Limitations can include: There can be an adequate visualization of the upper thoracic spine and potentially confounding spinal diseases. The diagnosis of osteoporosis using BMD relies largely on the relationship between BMD and fracture risk (set by World Health Organization, WHO), which was achieved based on data for postmenopausal Caucasian women, and is only

applicable to DEXA assessments at the spine, hip, and forearm (no other sites). Thus, the precise relationship between bone mineral density and fracture risk in younger, premenopausal women is currently not clear. Nonetheless, the above relationships had at least a moderate correlation coefficient, but did not reach statistical significance, likely due to low sample size. Thus, it is likely that larger sample sizes are necessary to further evaluate these relationships.

3.4 AMS analysis of collagen extracted from human bones by NaOH method

The amounts of Hyp extracted from chicken, cow or human bone collagen were insufficient for AMS analysis, as described in previous sections; thus, collagen form two human bones, which are previously used for forensic purpose, were analyzed as a test. Collagen was extracted from these bones by NaOH method and then subjected to AMS analysis, in which ^{14}C content was measured. The ^{14}C content obtained was then compared with the ^{14}C calibration curve, a so-called “Bomb Test Pulse” (Fig. 16), and the estimated year of birth of human, whose bones were used, were successfully obtained (Table 10).

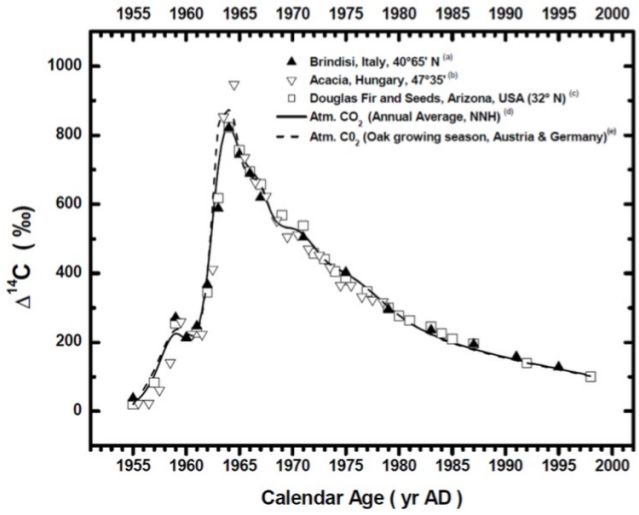


Figure 16 ^{14}C Calibration curve – "Bomb Test Pulse"

(Image was adopted from Quarta et al., 2005).

Table 10 Preliminary AMS analysis of collagen extracted from human bones by NaOH method.

Bone ID	$\Delta^{14}C$	±	Cal AD
AB2	1.2143	0.0053	1959 - 1961 or 1983 - 1985
AB3	1.2133	0.0053	1959 - 1961 or 1983 - 1985

These results showed that the two bones contain ^{14}C contents of between $\Delta^{14}C$ = 1.2143 and 1.2133, and the two individuals have the same estimated year of birth between 1959 and 1961, or between 1983 and 1985.

Chapter 4

Conclusions and Future works

This work presents the determination of Hyp in bone collagen extracted by three different methods (NaOH, KOH, and HCl methods) in conjunction with LC-MS analysis, the preliminary EA that can be used to obtain the carbon content in the collagen and the preliminary AMS analysis of collagen extracted from forensic human bones by NaOH method.

Base on the results obtained, the following can be concluded:

(1) Among the three extraction methods, KOH is the best extraction method that results in highest Hyp yield.

(2) KOH method was successful be employed to extract Hyp from all bones used in this work: chicken, cow, and human bones.

(3) Preliminary EA experiment indicated that the extracted collagen contained enough ^{14}C for a good measurement.

(4) Although the LC-MS procedure did not provide a sample of Hyp that could be further analyzed by AMS, it was shown that the use of a Preparatory HPLC would have provide sufficient material for AMS analysis had it been available.

(5) Comparing the content of Hyp in bones of osteoarthritic patients with different demographics indicated that there may be a relationship between the bone turnover (thus different Hyp content) and risk for osteoarthritic progression. This suggests that the determination of Hyp excrete in urine, in conjunction with ^{14}C-dating method, may be used as a novel biomarker for osteoarthritis.

(6) The preliminary AMS analysis indicated that NaOH extraction method is able to extract collagen from human bones with amount that is sufficient for AMS analysis, thus can be used for collagen extraction, in addition to KOH method, in future experiments.

The work provides additional information on the best extraction method for Hyp/collagen that has the potential for significantly benefiting its analysis by accelerator mass spectrometry (AMS) in the medical community to get information about bone disease.

Nonetheless, the following future works can be carried out:

(1) Further experiments may be conducted using more advanced analysis methods that can detect a trace amount of Hyp.
(2) Preparative HPLC may be used in future experiments to obtain higher amount of Hyp at the level that is suitable for AMS analysis.
(3) Based on the data, which suggests that bone turnover correlates with Hyp content, this content has a relationship with osteoarthritic progression. Further experiments should be conducted by using larger numbers of patients with larger demographical variant to confirm the relationship.
(4) Future AMS methods to analyze the ^{14}C content from Hyp in urine need to be developed to use this as a bio-marker for diseased bones, such as osteoporotic and osteoarthritic bones.

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Appendix

1. List of chemicals and instruments used

Chemicals and instruments, other than those described in methods section, used in this study are listed in Tables A.1 and A.2, respectively.

Table A.1 Chemicals used in this study.

Chemical	Physical state	Supplier
KOH	Solid	Fisher Scientific
HCl	Liquid	Fisher Scientific
NaOH	Solid	Fisher Scientific
Saline solution	Liquid	Waters Inc.
Hydroxyproline	Solid	Sigma Aldrich
Formic acid	Liquid	Fisher Scientific

Table A.2 Instruments used in this study.

Instrument	Supplier	Purpose
Centrifuge	Fisher Scientific	Separate solid and liquid samples
Freeze dryer	Scientific	Dry collagen
Balance	Mettler Tolled	Weigh chemicals
Fume hood	Ottawa lab	Handling chemicals
Hammer	Ottawa market	Crush bones
Coffee grinder machine	Ottawa market	Grind bones
Oven	Vinic Technologies	Dry bones
Test tube	Falcon	N/A
Nylon Filter	Sterlitech	Filter samples

2. Data for construction of the LC-MS calibration curve

In the construction of calibration, standard Hyp with varying mass were prepared and analyzed by HPLC. The mass of standard Hyp and their corresponding HPLC peak area used for the construction of calibration curve are shown in Table A3, and the calibration curve is shown in Fig. A1.

Table A3 Data for calibration curve.

Hyp standard	Mass of Hyp (mg)	LC peak area
Dil2	0.014	3.30E+06
Dil3	0.0014	4.40E+05
Dil4	0.00014	5.40E+04

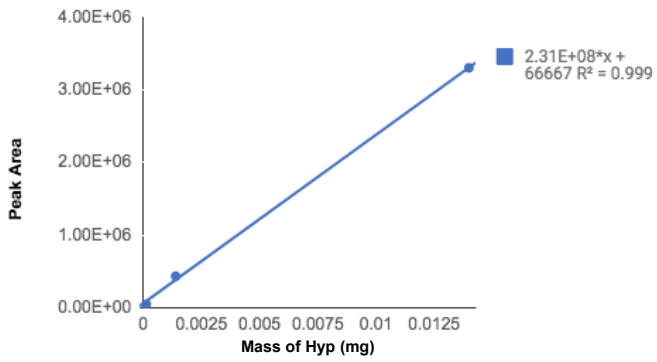

Figure A1 Calibration curve for the determination of Hyp content obtained by LC analysis.

3. Raw data from preliminary elemental analysis (EA)

Table A4 Raw data from EA of collagen extracted from chicken bone by NaOH and KOH methods.

Est. Age	Lab ID	Client ID	Sample Type	Mat Code	Mass (mg)	Peak CO_2 (uV)	Compound % N	Compound % C	C:N	mgC
Modern	n/a	Chicken – KOH	Bone	n/a	2.521	267000	4.94	30.28	5.25	0.76
Modern	n/a	Chicken – NaOH	Bone	n/a	2.343	374000	15.88	45.46	2.45	1.07

4. Raw data for Hyp in collagen extracted from chicken and cow bones

Table A5 Raw data for Hyp in collagen extracted from chicken bone by different methods.

Extraction method	Fresh bone (g)	Dried bone (g)	Dried collagen for LCMS (mg)	SAMPLE TYPE	Injection volume	Ug of hyp	Ug of HYP / g collagen	Ug of C from hyp/g collagen
KOH	1.299	1.037	26.52	n/a	1 ug	38.900	1466.82	61.5361
NaOH	2.26	0.070	20.65	n/a	1 ug	0.000	0.00	0.00000
HCl	9.99	6.934	31.34	n/a	1 ug	4.170	133.06	5.5820

Table A6 Raw data for Hyp in collagen extracted from cow bone by KOH method.

Extraction method	Fresh bone (g)	Bone with lipid removed (g)	Collagen retrieved (g)	Collagen selected for LP (mg)	HYP area in HPLC	HYP separated (ug)	Amount of carbon (ug)
KOH	500	80	4.6	4600	2.89E+06	1039.26	475.98

5. Raw data for Hyp in collagen extracted from human bones

Table A7 Raw data for Hyp in collagen extracted from (human) bones of patients with different demographics by KOH method.

Patient ID	Sex	Time between Rx and Surgery (months)	BMI	Age	K&L Grade	L1 to L2		Femoral Neck		Total Hip		Bone with lipids removed (g)	Collagen obtained (g)	Collagen used (mg)	Area of HPLC peak	Mass of HYP in HPLC peak (µg)	Mass of carbon in HPLC peak (µg)	Mass of C obtainable if all collagen used (µg)
						BMD g/cm2	T-Score	BMD g/cm2	T-Score	BMD g/cm2	T-Score							
H007 (Human 3)	Male	18	24.4	73	3	BMD not completed, diagnosed with diffuse osteopenia						3.833	2.737	28	0	-0.03169601089	-0.01451604833	-1.418943725
H008 (Human 1)	Female	9	25.5	70	4	0.882	-2.4	1.062	0.2	0.936	-0.6	3.29	2.9	30.89	220000	0.6749861072	0.3091282052	29.02142425
H009 (Human 4)	Male	8	27.3	65	3			1.008	-0.5	1.119	0.7	3.74	2.44	29.42	40000	0.095435519	0.0437072858	3.624941446
H010 (Human 2)	Female	36	22.8	70	4	1.013	-1.4	1.137	0.7	1.055	0.4	3.13	2.01	29.65	10000	0.00003174605008	0.00001453896514	0.0009856094412

Table A8 Output from linear regression of Hyp data in collagen from human bone, from which Pearson Correlation and P-values were given.

Correlations

		HYP_mass_mcg	Sex	Disease_duration_ months	BMI	Age	KL_Grade	FemNeckBMD	FemNeckT_score	HipBMD	HipT_score	HipZ_Score	Carbon_mass_mcg
Pearson Correlation	HYP_mass_mcg	1.000	.383	-.584	.243	.383	.383	-.223	-.035	-.759	-.938	-.898	1.000
Sig. (1-tailed)	HYP_mass_mcg	.	.375	.302	.422	.375	.375	.428	.489	.226	.112	.145	.000

Table A9 Output from linear regression of Hyp data in collagen from human bone using Spearman's correlations, which often used for smaller sample sizes and non-parametric data.

Correlations

			Sex	Disease_duration_ months	BMI	Age	KL_Grade	FemNeckBMD	FemNeckT_score	HipBMD	HipT_score	HipZ_Score	HYP_mass_mcg	Carbon_mass_mcg
Spearman's rho														
	HYP_mass_mcg	Correlation Coefficient	.447	-.600	.600	-.632	.447	-.500	-.500	-.500	-.500	-1.000**	1.000	1.000**
		Sig. (2-tailed)	.553	.400	.400	.368	.553	.667	.667	.667	.667	.	.	.
		N	4	4	4	4	4	3	3	3	3	3	4	4

**. Correlation is significant at the 0.01 level (2-tailed).

6. Raw data for AMS analysis of collagen extracted from human bones (used for forensic purposes)

Lab ID	Submitter ID	Material	Mat. Code	$F^{14}C$	±	cal AD
UOC-4227	AB2	bone	B	1.2143	0.0053	1959–1961 or 1983–1985
UOC-4228	AB3	bone	B	1.2133	0.0053	1959–1961 or 1983–1985