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Quantitation of hydroxyproline in bone by gas chromatography–mass spectrometry

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Abstract

A validated gas chromatography (GC)–mass spectrometric (MS) method for the analysis of hydroxyproline in rat femur is reported. Hydroxyproline in bone hydrolysates was extracted with an anion exchange resin and the N(O)-*tert*-butyldimethylsilyl derivatives analyzed by GC–MS. The hydroxyproline concentration was estimated relative to pipecolic acid, 3,4-dehydroproline and *n*-tetracosane as internal standards. The mass-to-charge ratios (*m*/*z*) for the ions used for quantitation by single ion monitoring were 314 *m*/*z* for hydroxyproline, 198 *m*/*z* for pipecolic acid, 256 *m*/*z* for dehydroproline and 57 *m*/*z* for n-tetracosane. A coefficient of variation of 5.8% was achieved and the limit of detection was calculated to be 0.233 μmol/l bone hydrolysate.

Keywords: Hydroxyproline

1. Introduction

Diseases involving the loss or breakdown of bone matrix represent a major healthcare problem [1,2]. Senile Osteoporosis affects an estimated one-third of women aged 60–70 and two-thirds of women aged 80 and older, resulting in approximately 200 million women worldwide suffering from this condition according to the International Osteoporosis Foundation (IOF) [3]. Osteoporosis may also occur secondary to chronic liver disease [4]. The consequences of osteoporosis include an increase in fracture risk of long bones and vertebrae that may result in morbidity and even mortality [2].

Type I collagen, the major protein component in bone, comprises over 90% of the organic bone matrix and is therefore important for the understanding of bone formation and turnover. Collagen fibers provide bone with tensile strength. The amino acid sequence of collagen is rich in proline. Approximately 50% of the proline side chains are hydroxylated post-translationally to form hydroxyproline (Hyp) [5–7]. During collagen breakdown, Hyp is released from bone and not recycled to form new collagen. Serum or urinary Hyp is therefore considered to be an osteoclastic bone resorption marker. A small fraction (10–20%) is excreted in the urine, while the remainder is transaminated in the liver [6–8].

Various analytical techniques have been employed to measure Hyp in urine, serum, and collagen-rich tissues for determination of bone composition, and to study the rate of bone resorption and collagen metabolism. Urinary Hyp, however, is not specific for bone collagen breakdown, since several other factors contribute significantly to the amount of Hyp found in urine [6,7]. These include collagen from skin and other tissues, dietary factors, newly synthesized collagen that is rapidly degraded, and the C1q fraction of the classical complement pathway, which is activated during acute inflammatory reactions [9]. The measurement of Hyp in bone is less prone to interference, and therefore more specific. Hyp measured in bone may thus reflect bone formation and therefore osteoblastic activity. It may be a very useful tool in the animal research setting.

Colorimetric methods employed for the measurement of Hyp involves the oxidation of hydrolysed Hyp with *n*-chloro-*para*-toluene sulfonamide sodium salt (chloramine-T) to form a pyrrole, which is subsequently extracted into

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toluene and reacted with Elrich's reagent to form a red chromophore ($\lambda = 560$ nm) [10–15]. Although this method is very specific for Hyp, it is time consuming and difficulty also lies in the control of the oxidation and color formation reactions [13,16].

High-performance liquid chromatography (HPLC) have been used extensively in the analysis of Hyp [17–23]. Amino acids are derivatized using a double derivatization reaction with *o*-phthaldialdehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC) or 9-fluorenylmethoxycarbonyl chloride. Alternatively, Tsuruta and Inoue [20] and Inoue et al. [21,22] have shown that 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride is also a very effective derivatization reagent for Hyp in serum and urine samples.

Gas chromatography (GC) with flame ionization detection (FID) have also been used for the detection of Hyp in various matrices [8,24–30]. A number of derivatization agents have been used to improve the chromatographic behavior of amino acids. The carboxylic acid-, amine-, and the aliphatic β-hydroxy functional groups of Hyp contain three kinds of protons that can be substituted by derivatization.

Reaction of amino acids with ethylchloroformate (ECF) is extremely reliable [27,28], however, Schilling et al. [24] and Hušek [27] reported that in the case of Hyp, reaction with ECF does not derivatize the β-hydroxy group. Esterification with *n*-butanol and subsequent trifluoroacetylation; however, derivatized Hyp completely, and blocked all three reactive sites [8,16,26].

GC–MS has also been used for the detection of Hyp in standard solutions [31,32], collagen hydrolysates [33], and cultured fibroblasts [34]. Various methods have been proposed for the derivatization of Hyp for GC–MS analyses. Woo and Lee [35] and Woo and Chang [36] successfully employed *N*-methyl-*N*-[*tert*-butyldimethylsilyl]-trifluoroacetamide (MTBSTFA) for the derivatization of amino acids in standard solutions, soybean and bovine serum albumin (BSA) hydrolysates. This silylation reagent derivatizes all three protons of Hyp simultaneously. The advantages of MTBSTFA above other silyl derivatives include enhanced reactivity towards functional groups, shorter reaction times, and increased resistance towards hydrolysis [35].

The following procedure describes the analysis of Hyp in rat femurs, using GC–MS detection after acid hydrolysis, extraction with an anion exchange resin and derivatization with MTBSTFA.

2. Experimental

2.1. Reagents and chemicals

Hydrochloric acid, acetic acid and sodium hydroxide pellets were purchased from Merck (Darmstadt, Germany). Chloroform, methanol, MTBSTFA, 4-hydroxyproline, pipecolic acid, and 3,4-dehydroproline were supplied by Fluka (Buchs, Switzerland). Acetonitrile were purchased from Ultrafine Unlimited (Finchley, London) and *n*-tetracosane ($C_{24}H_{50}$) was purchased from Analabs Inc. (No. Haven Connecticut, USA). BioRad Laboratories (Hercules, CA, USA) supplied the AG MP-1 anion exchange resin (100–200 mesh, chloride form). De-ionized water from a Milli-Q Reagent Water System was used in all standard and sample preparation procedures.

2.2. Standard stock solutions

All standards were dissolved in deionized water, except for $C_{24}H_{50}$, which was dissolved in chloroform. Concentrations of the standard and internal standard stock solutions were as follows: Hyp 763 μmol/l; 3,4-dehydroproline 885 μmol/l; pipecolic acid 775 μmol/l; $C_{24}H_{50}$ 2000 μmol/l.

2.3. Equipment

A HP6890 GC system fitted with a HP7683 Auto injector and a HP5973 mass-selective detector (MSD) (Agilent Technologies, Palo Alto, CA, USA) were used for GC–MS analysis. Data collection and integration were performed with HP Chem Station software. A DB-5MS (J&W Scientific, Agilent Technologies, Palo Alto, CA, USA), capillary column was used (30 m × 250 μm; d_f 0.1 μm). Tuning of the MSD was performed with perfluorotributylamine (PFTBA) for masses 69, 219, and 502 using the autotune option.

2.4. Hydrolysis of femur samples

Rat femurs were cleaned completely of all soft tissue and dried in an oven at 100 °C for 4 h. The dry weight of each femur was recorded. The femurs were then hydrolyzed with HCl (10 ml; 6N) in tightly capped glass vials at 100 °C for 24 h. The hydrolyzed bone samples were centrifuged for 5 min (4000 rpm at 4 °C).

2.5. Sample preparation

An aliquot (30 μl) of the supernatant, from the hydrolyzed bone samples, was transferred to a test tube. Pipecolic acid stock solution (640 μl), 3,4-dehydroproline stock solution (1280 μl), and diluted sodium hydroxide solution (4450 μl; 0.005 M) were added to give a final volume of 6400 μl. The sodium hydroxide was used to adjust the pH to 12. After the sample was vortexed for 1 min, an aliquot (3 ml) was applied to the anion exchange resin column to extract the analytes.

2.6. Extraction procedure

Equal amounts of glass wool were inserted into Pasteur pipettes and anion exchange resin (200 mg) was applied to each column in 6 ml water. The resin was rinsed with methanol (3 ml) and water (3 ml) successively, after which the sample solution (3 ml) was applied to the column. After application of the sample, the column was rinsed again

with water (3 ml), followed by methanol (3 ml). The analytes were eluted with an acetic acid in methanol solution (1.5 ml, 2N), $C_{24}H_{50}$ stock solution (11 μl) was added and the mixture was vortexed for 30 s. The eluates were dried completely under a stream of dry nitrogen, and resolvated in the methanolic acetic acid solution (300 μl, 2N). The samples were vortexed thoroughly for 1 min to ensure complete solvation.

2.7. Derivatization procedure

The derivatization procedure described by Woo and Lee [35] was adapted to prepare the N(O)-*tert*-butyldimethylsilyl derivatives of the amino acids. In short, an aliquot of the up-concentrated sample (200 μl) was transferred to the insert of a 2 ml GC injection vial and dried completely under dry nitrogen for at least 90 min. Acetonitrile (30 μl) and MTBSTFA (50 μl) were added to the dried sample, the vial was capped with a Teflon lined cap and vortexed for 30 s. After incubation at 90 °C for 1 h, the sample was allowed to cool down to room temperature. The derivatized samples were stored at −78 °C until analyses.

2.8. Gas chromatographic–mass spectrometric (GC–MS) detection procedure

A 2 μl volume of the derivatized sample was injected in split mode with a split ratio of 50:1. The inlet temperature was set at 250 °C and helium carrier at a constant flow-rate of 2 ml/min (51 cm/s). The oven temperature program had an initial isotherm of 44 °C for 10 min, after which it was ramped to 190 °C at a rate of 8 °C/min. A second isotherm followed at 190 °C for 6 min, after which it was ramped to 300 °C at a rate of 20 °C/min. The total chromatographic time was 39.75 min. The transfer line temperature was set at 280 °C and that of the quadrupole at 106 °C. The source temperature was 230 °C. A solvent delay time of 20 min was used to allow for solvent elution before the source was turned on. All mass spectra were recorded at 70 eV. Chromatograms were recorded in the scan mode (50–500 *m*/*z*) initially, to identify the analytes in standard solutions and to ascertain their retention times. Quantitation was performed in the single ion monitoring (SIM) mode with a dwell time of 100 ms. The mass-to-charge ratios (*m*/*z*) of the ions that were used for (SIM) quantitation were: 314 *m*/*z* for hydroxyproline, 198 *m*/*z* for pipecolic acid, 256 *m*/*z* for 3,4-dehydroproline and 57 *m*/*z* for $C_{24}H_{50}$.

2.9. Calibration curves

Calibration standards were prepared by using the work-up procedure outlined earlier. The method of standard addition was used to prepare the calibration standards for Hyp with the following concentrations: 15.2, 45.8, 76.3, 115 and 137 μmol/l. To simulate the matrix, bone hydrolysate (6 μl, in a final volume of 6400 μl) was added to each calibration standard. 3,4-Dehydroproline (1280 μl from stock solution) and pipecolic acid (640 μl from stock solution) were added as internal standards. $C_{24}H_{50}$ (11 μl from stock solution) was also added as an internal standard, after anion exchange clean-up.

3. Results and discussion

A chromatogram of calibration standard 3 recorded in both SCAN and SIM modes are shown in Fig. 1. The retention times for 3,4-dehydroproline, pipecolic acid, Hyp and $C_{24}H_{50}$ were 25.28, 26.48, 31.30 and 36.37 min, respectively.

The electron impact spectra of the N(O)-*tert*-butyldimethylsilyl derivatives of Hyp, pipecolic acid and 3,4-dehydroproline are shown in Fig. 2. The abundance of the molecular ions for the derivatives of Hyp (473 *m*/*z*), pipecolic acid (357 *m*/*z*) and 3,4-dehydroproline (341 *m*/*z*) were low. The base peaks, 314 *m*/*z* and 198 *m*/*z*, were used for SIM quantitation for Hyp and pipecolic acid derivatives, respectively. The base peak 182 *m*/*z* for the 3,4-dehydroproline derivative could not be used since an unknown compound, overlapping partially with the 3,4-dehydroproline chromatographic peak, also proved to have a strong abundance of 182 *m*/*z*. According to literature [36,37], the *tert*-butyldimethylsilyl derivatives of amino acids show a $M^+ - 85$ ion, therefore the characteristic 256 *m*/*z* ($M^+ - 85$) ion, was used for SIM quantitation. Selection of this ion enhanced the specificity for 3,4-dehydroproline, however, due to its low abundance, sensitivity was compromised.

3.1. Sample preparation

Hydroxyproline, pipecolic acid and 3,4-dehydroproline have to be converted to volatile derivatives prior to GC analysis. The ethoxycarbonyl derivatives as described by Hušek [27] were initially tested. It was found that this procedure did not derivatize the aliphatic hydroxy proton, resulting in peak tailing, poor resolution and an increased detection limit. It was therefore decided to utilize the N(O)-*tert*-butyldimethylsilyl derivatives since MTBSTFA is sufficiently reactive to derivatize not only the carboxylic acid and amine protons, but also the aliphatic hydroxy proton. Fig. 3 shows chromatograms of the level 3 calibration standard, with and without the use of anion exchange extraction. It can be seen that the anion exchange resin effectively extracted and up-concentrated the analytes. It also "cleaned" the sample from other interferents.

Initially, 3,4-dehydroproline and $C_{24}H_{50}$ were used as the internal standards. The use of 3,4-dehydroproline as the internal standard proved to be problematic since no repeatability in results could be achieved ($r^2 = 0.587$). Unlike previous reports [17,21–23,38], 3,4-dehydroproline was found not to be a suitable internal standard for gas chromatographic Hyp quantitation. This may be ascribed

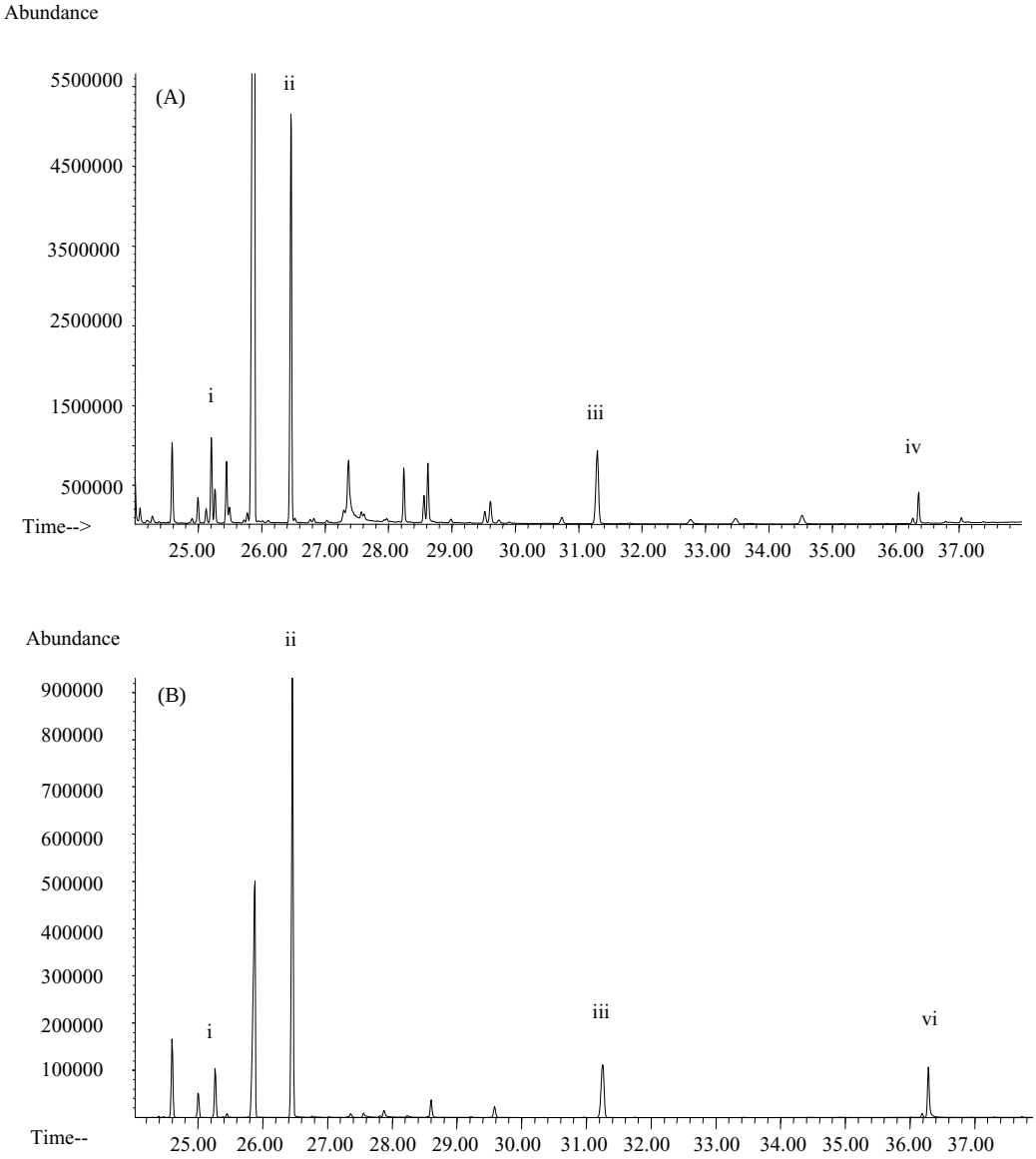

Fig. 1. Chromatogram recorded in (A) SCAN mode (*m*/*z* 50–500) and (B) chromatogram recorded in SIM mode. The elution order is as follows: (i) 3,4-dehydroproline, (ii) pipecolic acid, (iii) 4-hydroxyproline, (iv) $C_{24}H_{50}$.

to the difference in reactivity of alkanes and alkenes at the elevated temperatures in the heated zones of the gas chromatographic apparatus, as well as the decreased sensitivity for 3,4-dehydroproline due to the choice of the 256 *m*/*z* ($M^+ - 85$) ion. The use of $C_{24}H_{50}$ as internal standard yielded better results ($r^2 = 0.966$), however, $C_{24}H_{50}$ is applied to the sample after anion exchange extraction, and therefore corrects only for sample losses during the up-concentration and derivatization steps. The use of pipecolic acid internal standard [33] showed dramatically improved results ($r^2 = 0.992$) as compared to 3,4-dehydroproline.

3.2. Assay validation

The calibration curves were found to be linear up to a concentration 0.14 mmol/l bone hydrolysate. This compared favorably with the results of GC methods for Hyp in literature. Since the proposed method utilizes mass spectrometric detection, the selectivity will be superior to other chromatographic methods, which rely on retention time identification only [26,30,38,39]. The linear regression data for Hyp relative to internal standards, pipecolic acid, dehydroproline and $C_{24}H_{50}$, are shown in Table 1. The within batch coefficient of variation (CV) was determined by analyzing five samples of calibration standard 3 during the same batch, whereas the between batch CV was determined by analyzing calibration standard 3 in five separate batches. The calculated detection limit for Hyp (3 × signal-to-noise) was 0.233 μmol/l bone hydrolysate.

It can be depicted from Table 1 that the use of pipecolic acid as internal standard provided more consistent results within and between batches, as compared to $C_{24}H_{50}$. A

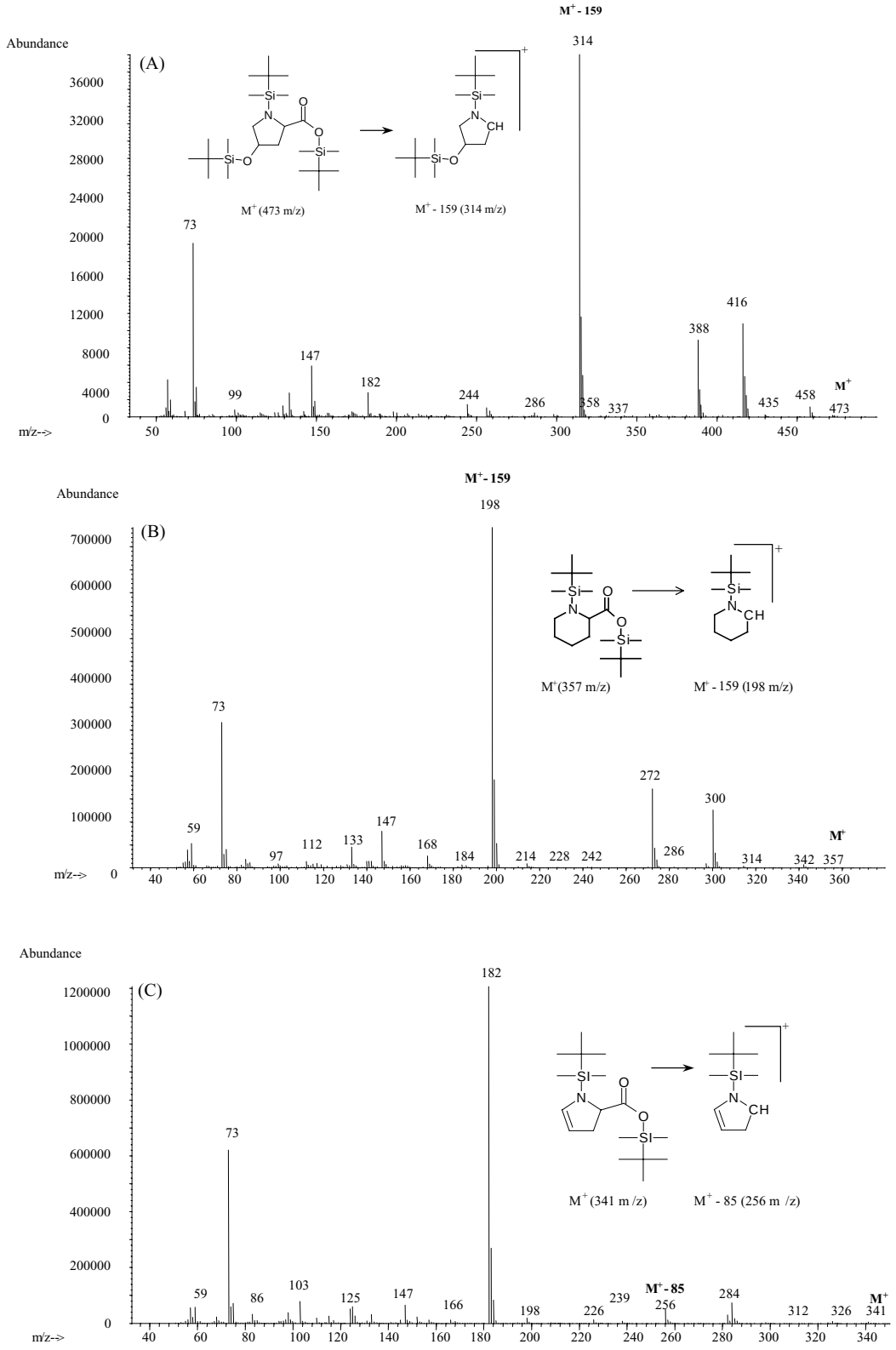

Fig. 2. Full scan mass spectra of the N(O)-*tert*-butyldimethylsilyl derivative of (A) 4-hydroxyproline, (B) pipecolic acid and (C) 3,4-dehydroproline.

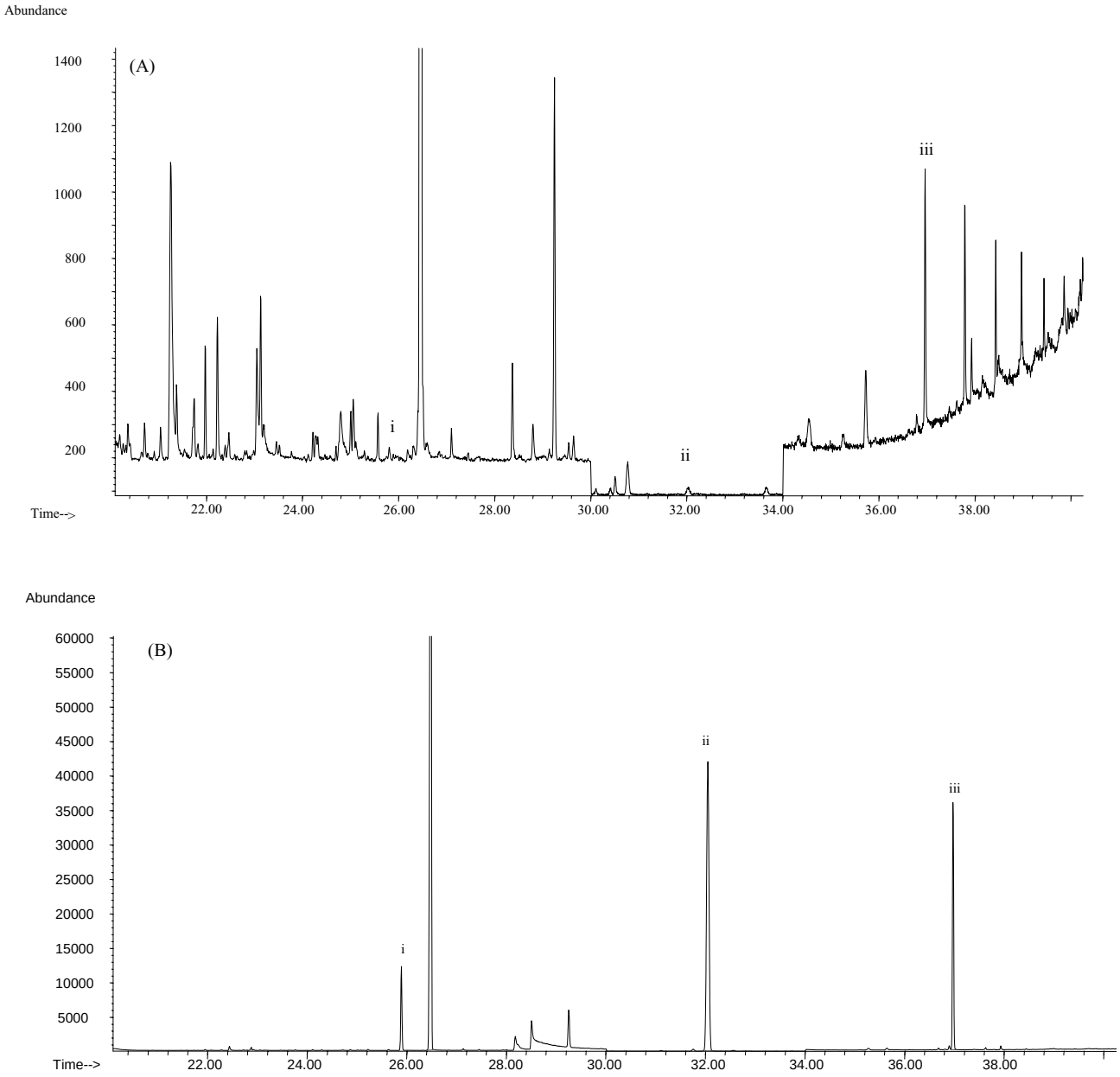

Fig. 3. Chromatogram of the level 3 calibration standard: (A) without and (B) with subjection to anion exchange extraction: (i) 3,4-dehydroproline, (ii) 4-hydroxyproline, (iii) $C_{24}H_{50}$.

coefficient of variation of 5.8% could be achieved by utilizing pipecolic acid as internal standard, 7.82% with $C_{24}H_{50}$ and a mere 54.1% with 3,4-dehydroproline. The enhanced performance of pipecolic acid as internal standard can be attributed to the fact that molecular structure of pipecolic acid resembles that of Hyp more closely than $C_{24}H_{50}$ and 3,4-dehydroproline. Pipecolic acid is also added at the beginning of the sample work-up procedure.

Table 1
Regression analysis parameters of the calibration curves utilizing 3,4-dehydroproline, pipecolic acid and $C_{24}H_{50}$ as internal standards

Internal standard	r^2	Slope (relative response factor (RRF), analyte/IS)	*Y*-intercept	CV (%) (within batch, $n = 5$)	CV (%) (between batch, $n = 5$)	Mean recovery (%) at standard 3 concentration level ($n = 5$)
3,4-Dehydroproline	0.5873	2.2794	1.2724	60.166	54.10	94.48
Pipecolic acid	0.992	0.0267	0.0324	6.28	5.79	96.96
$C_{24}H_{50}$	0.9656	0.4383	0.3252	7.88	7.82	92.68

4. Conclusions

This sensitive and selective gas chromatographic mass spectrometric assay is an appropriate method for quantitation of hydroxyproline in bone. It replaces a combination of derivatization procedures by a simpler one-step silylation procedure. In the case of the ethoxycarbonyl derivatization procedure, the non-derivatized aliphatic proton rendered quantitation unreliable due to the poor chromatographic properties of the derivatized Hyp. The employment of an anion-exchange resin effectively extracted and cleaned the sample and enhanced the assay selectivity. The use of pipecolic acid as internal standard, compared to 3,4-dehydroproline, improved the reliability of the assay.

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