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Review

# Simple and sensitive method for quantification of fluorescent enzymatic mature and senescent crosslinks of collagen in bone hydrolysate using single-column high performance liquid chromatography

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ABSTRACT

A rapid high performance liquid chromatographic method was developed including an internal standard for the measurement of mature and senescent crosslinks concentration in non-demineralized bone hydrolysates. To avoid the demineralization which is a tedious step, we developed a method based on the use of a solid-phase extraction procedure to clean-up the samples. It resulted in sensitive and accurate measurements: the detection limits as low as 0.2 pmol for the pyridimium crosslinks and 0.02 pmol for the pentosidine. The inter- and intra-assay coefficients of variation were as low as 5% and 2%, respectively, for all crosslinks.



**Contents**



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## 1. Introduction

Crosslinks of collagen play an important role in bone strength and mechanic stability of the collagen network. They are significant molecules in several kinds of connective tissue abnormalities and age-dependent modifications. Crosslinks with fluorescent properties are derived from two different pathways: those initiated by the enzyme lysyl oxidase and those derived from the non-enzymatic glycation [1–3]. The enzymatic process leads to the formation of mature trivalent crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD) that stabilize the collagen fibers. On the other hand, the formation of advanced glycation end products (AGEs) has been described in type I collagen which results in the accumulation of reducing sugars in bone tissue. The most widely studied AGE is pentosidine (PEN). This senescent molecule is a pentose-derived fluorescent crosslink formed between lysine and arginine residues in collagen. The formation and accumulation of AGEs increases with aging and, particularly in tissues characterized by a low turnover such as bone or cartilage, but this also can occur in patients with diabetes mellitus [4,5].

Since PYD, DPD and PEN are representative crosslinks having different characteristics, we found it beneficial to measure them in connective tissues for elucidating their roles. PYD and DPD are generally determined separately by sensitive fluorescent detection on prefractionation with CF-1 cellulose using preponderantly *n*-butanol as washing buffer to remove interfering fluorophores prior to high performance liquid chromatography (HPLC) using a reversed phase column [6]. Unfortunately this method was not suitable for PEN quantification [7,8]. Takahashi et al. [7] had previously reported a method to quantify PYD, DPD and PEN in tissue hydrolysates which required a SP-Sephadex C25 pre-fractionation step (instead of CF-1 cellulose). However, this cation exchange procedure presented a disadvantage due to a required concentration step before HPLC analysis. It has also been reported that direct crosslinks measurement methods in hydrolysate of tissues can be measured using reversed phase HPLC, however, the samples are contaminated which contributed to decrease the life-time of the HPLC column [9]. Moreover, in almost all techniques described, to avoid any bad chromatography (i.e. baseline shift and undesirable peak shape), it is necessary to demineralize bone, which corresponds to a laborious step before the quantification of the crosslinks [8,9]. These methods are insufficient for routine analyses of bone tissues due to long analysis times and the lack of accuracy for quantification because no internal standard (INT STD) was used in almost all of the methods described [8]. Therefore, it is necessary to develop a quick and reproducible method for crosslinks measurements.

The present paper describes the development and validation of a rapid HPLC method including a commercial INT STD, which is suitable for the measurement of mature and senescent crosslinks concentration in non-demineralized bone hydrolysate. The developed method is based on the employment of a solid-phase extraction (SPE) procedure for the bone hydrolysate pre-treatment in order to remove interfering fluorochromes and to increase the life-time of the HPLC column.

## 2. Materials and methods

### 2.1. Chemicals

HPLC-grade acetonitrile and acetic acid were purchased from Carlo Erba-SDS (Val de Reuil, France) and heptafluorobutyric acid (HFBA) was purchased from Apollo Scientific Ltd. (Stockport, UK). 18 Ω water was purified using Purelab UHQ (Veolia Water STI, Anthony, France). Hydroxyproline HPLC kits were purchased from Bio-Rad (Hercules, CA, USA). Solid-phase extraction Chromabond® Crosslinks columns were supplied by Macherey-Nagel (Düren, Germany), internal standard was obtained from Bio-Rad (Hercules, CA, USA), PYD and DPD calibrator was purchased from Quidel Corporation (San Diego, CA, USA). PEN standard were synthesized as described previously [10] and calibrated against PEN standard kindly donated by Professor Takahashi.

### 2.2. Chromatographic conditions to quantify collagen crosslinks

#### 2.2.1. Tissue sample preparation

In order to validate our new HPLC method, we compared the crosslinks measurements in bone crude hydrolysate to the bone collagen hydrolysate (Fig. 1). To set up this method cortical calf bone was incubated with 0.2 M D-Ribose in PBS at 37 °C for 5 days in order to induce the formation of PEN *in vitro*, as previously described. Bone samples were powdered in liquid nitrogen-cooled freezer mill (Spex Centriprep, Metuchen, USA) and defatted in methanol/chloroform, extensively washed with deionized water and finally lyophilized. One part of the bone powder was demineralized according to the standard demineralization protocols. Briefly, the bone powder is cleaned by saline solution (2 M NaCl, 50 mM Tris–HCl, 20 mM EDTA, pH 7.5) containing protease inhibitors (complete protease inhibitor cocktail tablets from Roche Diagnostics, Basel, Switzerland and pepstatin 1 μg/mL from Sigma–Aldrich, Saint Quentin Fallavier, France) and then the bone powder was demineralized. Three different demineralization processes were tested in parallel: (i) 0.5 M EDTA in 0.05 M Tris buffer, pH 7.4, (ii) 4 M guanidine–0.5 M EDTA in 0.05 M Tris buffer, pH 7.4 and (iii) formic acid (1 M sodium citrate solution containing 45% formic acid). The demineralization step continued for 48 h at 4 °C, with daily changing of the demineralization solution. These solutions contained the same protease cocktail inhibitors as previously described to avoid any protein degradation. Demineralized bone powders obtained were extensively washed with deionized water and freeze-dried. In parallel, non-demineralized bone powders from each sample was directly hydrolyzed to measure collagen crosslinks (PYD, DPD and PEN).

#### 2.2.2. Acid hydrolysis of the samples and hydroxyproline measurement

Non-demineralized and demineralized powdered bone samples were hydrolyzed in 6N hydrochloric acid at 110 °C for 20 h in sealed glass tubes (10 mg of non-demineralized bone per ml or 1 mg of demineralized bone per ml). Hydroxyproline content was measured with an HPLC assay developed by Bio-Rad (Hercules, CA, USA) using an aliquot of sample hydrolysates. The amount was calculated assuming 300 nmol of hydroxyproline in 1 mol of collagen.

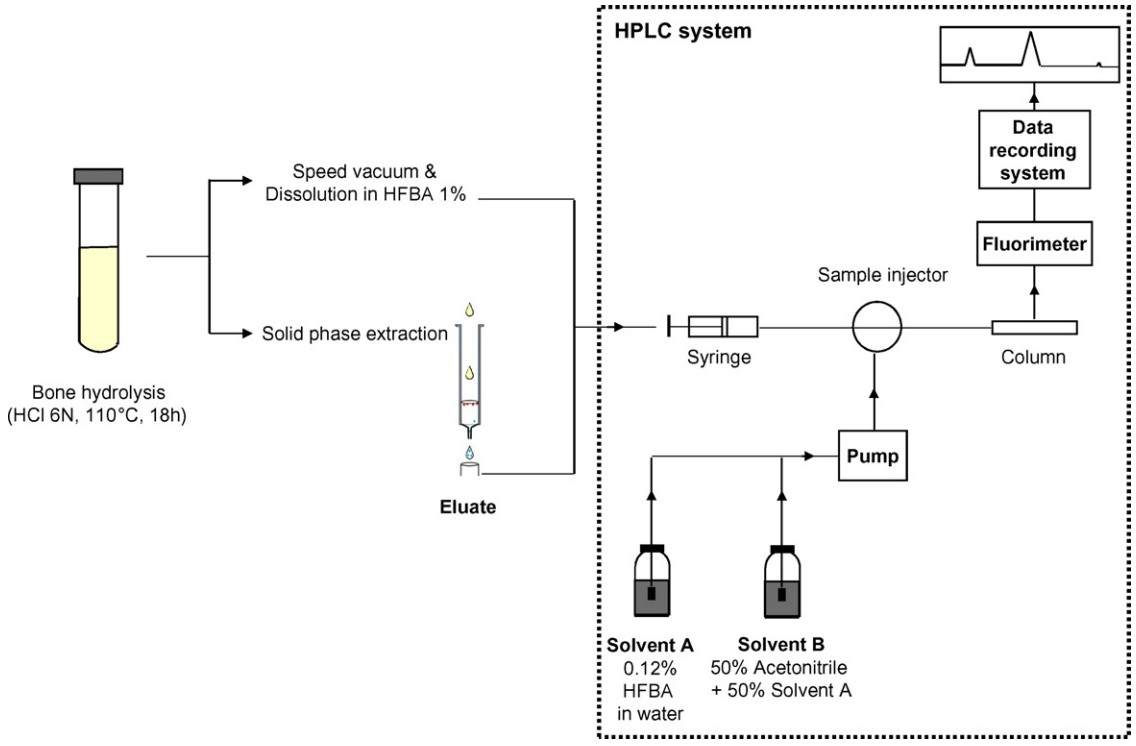


**Fig. 1.** Schematic drawing of the set up of the HPLC system: after acidic hydrolysis, collagen crosslinks in demineralized bone hydrolysate and in crude bone hydrolysate were quantified in parallel after direct injection on the HPLC system or after treatment on the SPE column. A concentration step is necessary before the direct injection on the HPLC.

### 2.3. Sample processing

#### 2.3.1. Sample pre-treatment

Bone hydrolysates were pre-fractioned on SPE Chromabond® Crosslinks to remove interfering fluorophores using a Vac Elut 20 Manifold apparatus (Varian, Inc., Palo Alto, CA, USA) and a N840FT.18 LABOPORT® pump (KNF Neuberger, Inc., Trenton, NJ, USA). Briefly, in 5 ml glassed tube, 60 μl of INT STD were added to 400 μl of hydrolysate samples and 2.8 ml of buffer composed of acetonitrile and acetic acid in a 6:1 ratio (v/v), respectively. After mixing, the solution was transferred to a SPE column previously placed inside a vacuum box and equilibrated with 2.5 ml of wash buffer prepared with acetonitrile, acid acetic and deionized water in the respective proportions 8:1:1 (v/v/v). 2.5 ml of wash buffer were used for rinsing the 5 ml glass tube and applied to the SPE column. Columns were extendedly washed with 4 × 2.5 ml of wash buffer to remove interfering fluorophores and 200 μl of deionized water were applied on the column. Finally, the columns were drained completely. Then crosslinks were eluted with 600 μl of 1% HFBA directly in 750 μl HPLC vials, mixed and then 60 μl were injected on the HPLC system [11,12]. Concerning direct injection of hydrolyzed bone samples on a HPLC reversed phase column, 100 μl of bone hydrolysates were concentrated using a Savant Speed Vacuum concentrator and reconstituted with 100 μl of 1% HFBA solution containing 15 μl of INT STD. Then 40 μl of sample was injected onto the HPLC system in order to have the same proportion of INT STD and bone hydrolysates pre-fractioned on SPE.

#### 2.3.2. HPLC conditions

PYD, DPD and PEN were separated by HPLC on an Alliance 2695 separation module equipped of with a 2647 Multi λ fluorescence detector and Empower2 chromatography data software (Waters Corp. Milford, MA, USA). Crosslinks were separated on an Atlantis dC18, 3 μm, 4.6 mm × 100 mm reversed phase column protected by an Atlantis dC18, 3 μm, 4.6 mm × 20 mm guard cartridge (Waters Corp., Milford, MA, USA). The column flow rate was 1.2 ml/min and the column temperature 40 °C. Briefly, molecules were separated by using a gradient solution. Solvent A consisted 0.12% of HFBA in 18 Ω pure water, and solvent B was 50% of solvent A and 50% of acetonitrile. The column was equilibrated with 14% solvent B prior to use. The separation of PYD and DPD was performed during the first 12 min of an isocratic step at 14% of solvent B, INT STD and PEN eluted during the following 24 min of gradient from 14 to 31% solvent B. PYD, DPD and the INT STD were monitored for fluorescence at an emission of 395 nm and an excitation of 297 nm and then wavelengths were shifted to 385 and 335 nm respectively for determination of PEN. Pyridinium crosslinks and PEN were quantified against calibrator and corrected by INT STD lost during all the preparation procedure. After each analysis, the column was rapidly washed with 100% acetonitrile for 2 min and equilibrated with solvent A [11,12]. The area of the peaks was used for the quantification of separated materials.

### 2.4. Validation procedures

#### 2.4.1. Extraction recovery

The extraction recovery of crosslinks on the SPE column was analyzed on non-demineralized bone hydrolysate pool under the two following conditions: (i) according to the procedure previously described in Section 2.3.1, 400 μl of bovine bone hydrolysate was spiked with defined amounts of PYD, DPD and PEN calibrators (337.5, 152.5 and 125 pmol/ml, respectively) and with 60 μl of INT STD. This solution was pre-treated on the SPE column and the molecules of interest were eluted with 600 μl 1% HFBA. Eluate, containing 6 μl of INT STD, 13.5, 6.1 and 5 pmol of PYD, DPD and PEN respectively, was injected onto the HPLC column or (ii) 400 μl of non-spiked bovine bone hydrolysate was pre-treated on the SPE column. Defined amounts of PYD, DPD, IS and PEN calibrators in a

**Table 1**
Effect of demineralization on crosslink amounts in bovine glycated bone. Cortical bovine bone was incubated with 0.2 M D-Ribose in PBS at 37 °C for 5 days in order to induce *in vitro* formation of PEN. The measurements were made on five different specimens after a SPE procedure.

| | PYD (mmol/mol coll) | DPD (mmol/mol coll) | PEN (mmol/mol coll) |
|---|---|---|---|
| No treatment | 133 (20) | 15 (2) | 131 (46) |
| Formic acid treatment (vs. no treatment) | 146 (23) [6.2 (4.8)] | 13 (2) [0.4 (0.8)] | 118 (37) [−8.6 (5.7)] |
| EDTA treatment (vs. no treatment) | 142 (23) [6.0 (2.5)] | 16 (2) [6.5 (4.4)] | 127 (43) [−3.0 (3.7)] |
| EDTA/Guanidine treatment (vs. no treatment) | 141 (20) [6.0 (4.3)] | 13 (2) [−13 (10)] | 143 (55) [9.2 (7)] |

*Note*: Results of the demineralization procedure on crosslink amounts are expressed as percentage of mean (standard deviation). The percentage change of bone crosslinks between demineralization treatments and crude bone hydrolysates were reported in square brackets in the table.

proportion similar to the one used for the first condition described above were directly added in 1% HFBA eluate. This preparation, containing 6 μl of INT STD, 13.5, 6.1 and 5 pmol of PYD, DPD and PEN, respectively, was injected onto the HPLC column. The two types of experiments were repeated 10 times. The recovery rates (RE %) were calculated using the following equation: RE (%) = response (bovine pool spiked with analytes before SPE treatment)/response (bovine pool spiked with analytes after SPE treatment) × 100.

#### 2.4.2. Linearity and calibration curve

Calibration standards were prepared and analyzed in triplicate. Calibration curves of each standard were analysed across an amount range of 0–1 nmol for PYD, 0–0.5 nmol for DPD and PEN and a relative amount range of 0–100% for INT STD (as the INT STD concentration was not provided by BioRad, we considered that the non-diluted commercial INT STD corresponds to 100%) to determine the fluorescence response linearity and then were fitted by square linear regression. To assess the linearity, deviations of the mean calculated amounts should be within ±15%. Moreover, the detection limit (signal-to-noise ratio = 4) was established.

#### 2.4.3. Reproducibility and precision

The reproducibility of the assay was assessed by repeated measurements of two different glycated bovine bone hydrolysate sample pools with low (pool A) and high (pool B) crosslink concentrations. Inter- and intra-assay precisions of the HPLC method were determined by assessing 10 replicates. Intra-assay precision was calculated from 10 injection runs of the same pre-fractioned bone sample pools and the inter-assay precision was determined by HPLC injections of 10 different pre-treated bone sample pools. The coefficient of variation (CV) was used as a measure for intra- and inter-assay precisions. Precisions should not exceed 15% CV.

### 2.5. Application

PYD, DPD and PEN were quantified in crude hydrolysate of cortical and trabecular bone of human lumbar vertebrae, in trabecular bone of dog lumbar vertebrae, sheep iliac crest and in cortical diaphysis femur of bovine, rat and chicken to validate this new method.

## 3. Results and discussion

### 3.1. Sample pre-treatment

For sample pre-treatment we focused on non-labor methods to accelerate sample processing. First we compared the amounts of collagen crosslinks (PYD, DPD and PEN) detected in non-extracted bone tissue hydrolysates with those demineralized with formic acid, EDTA or by an EDTA/guanidine treatment. For this analysis, cortical bovine bone was incubated with 0.2 M D-Ribose in PBS at 37 °C for 5 days in order to induce the formation of PEN *in vitro*. We found that the amounts in molecules of PYD, DPD and PEN were similar between non-extracted bone tissue and those demineralized with EDTA suggesting that all these methods were suitable for the measurement of collagen crosslinks (Table 1). Results obtained with Guanidine/EDTA and formic acid extraction showed poor reproducibility for DPD quantification due to a slight contamination (Fig. 2).

Representative chromatograms of calibrator and glycated bovine bone samples are shown in Fig. 2. With our HPLC method, we analyzed the pattern of non-demineralized and demineralized injected directly on the reverse phase HPLC column or after a SPE pre-treatment. It was observed that the patter noise is more important and interferes with the peaks of interest when the sample is injected directly onto the column (Fig. 2B–E). Moreover, the column performance decreased after about one hundred injections. Column degradation was evident by reduced sensitivity. This could be explained by the relatively dirty hydrolysates that were injected onto the column.

With the pre-treated procedure on column SPE Chromabond® Crosslinks, all the molecules were well separated, no contamination was observed in non-demineralized and in demineralized bone extracts (Fig. 2F, G, H and I). This simple sample clean-up procedure was found to yield sufficiently clean hydrolysates to allow proper quantification of collagen crosslinks and to allow a reasonable HPLC column life-time. The most important point concerning the SPE treatment is the reproducibility of the retention time compared to the direct injection method. Moreover, this novel approach decreases the time for quantification of the collagen crosslinks since it is not necessary to demineralize the bone and concentrate samples before injection on the HPLC system such as described in previous methods.

### 3.2. Chromatography

Over the past few years, the previously described methods required complicated sample preparation procedures with multiple steps that could easily contribute to a source of error within the method. This is why we developed a new method by which to measure these crosslinks by a single reversed-phase HPLC including an INT STD, after a quick and easy sample pre-treatment. This is the first method which used a pre-packed SPE Chromabond®

**Fig. 2.** Chromatograms obtained from glycated calf bone hydrolysates. Bones were incubated for 5 days in 0.2 M ribose to induce the formation of AGEs. Chromatograms of a calibrator (A) the amount of calibrators PYD, DPD and PEN for panel A are 8.41, 3.64 and 2.8 pmol, respectively. Chromatograms corresponding to direct injection are presented on the left panel, and the chromatograms corresponding to SPE treatment on the right panel. The chromatograms B and F correspond to the crude bone extract, the chromatograms C and G correspond to the demineralized bone with EDTA, the chromatograms D and H correspond to demineralized bone with EDTA/guanidine and the chromatograms E and I correspond to demineralized bone with formic acid. *Note*: Fluorescence was monitored with excitation at 297 and emission at 395 nm and was changed at 28 min to excitation at 335 nm and emission at 385 nm after elution of PYD, DPD and INT STD.

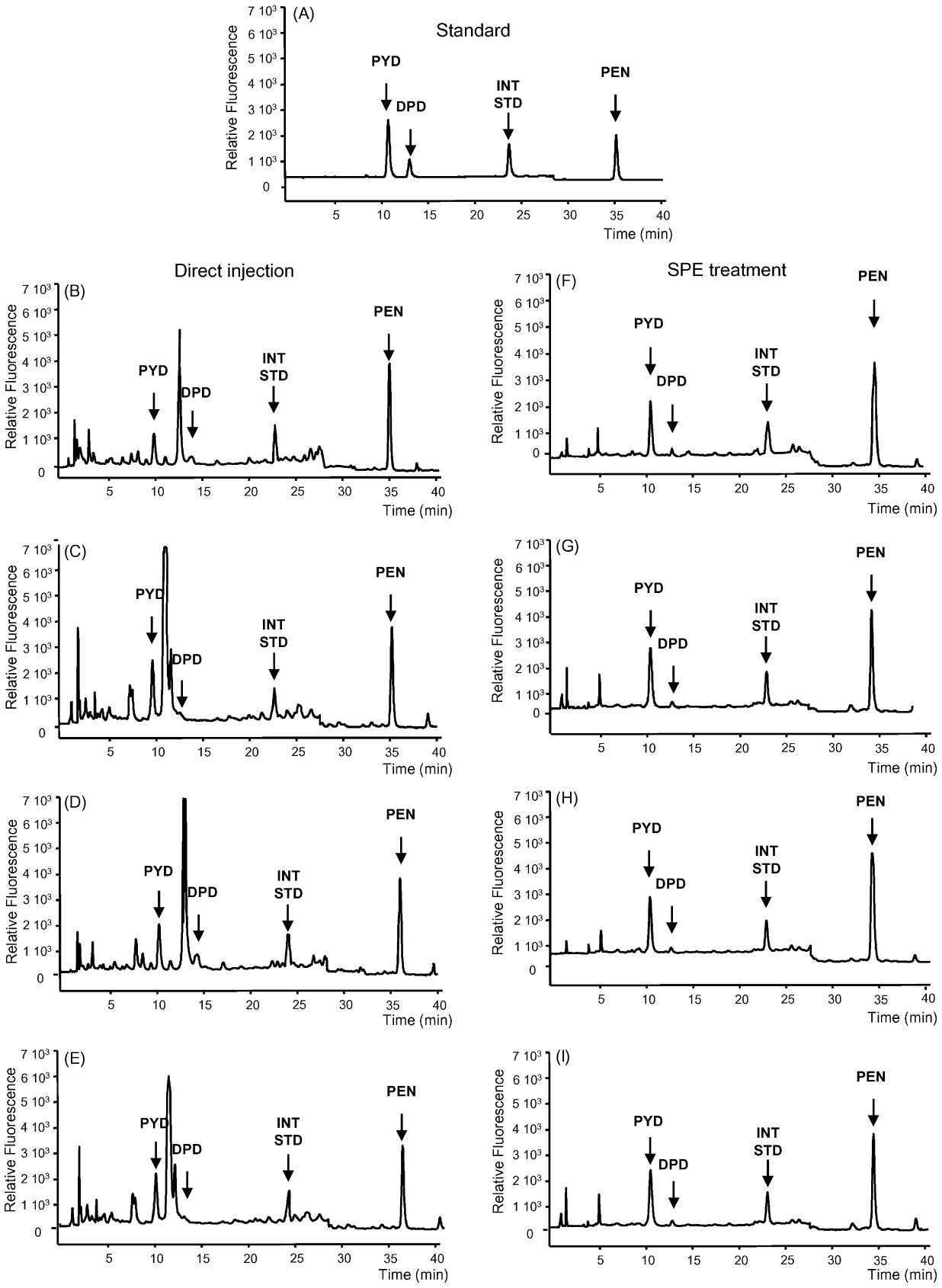
(A)
Standard
PYD
DPD
INT STD
PEN
Relative Fluorescence
Time (min)
Direct injection
(B)
(C)
(D)
(E)
SPE treatment
(F)
(G)
(H)
(I)

**Table 2**
Reproducibility of the measured crosslinks.

| | | Concentration (μmol/L) (mean (SD)) | | | Coefficient of variation (%) | | |
|---|---|---|---|---|---|---|---|
| | | PYD | DPD | PEN | PYD | DPD | PEN |
| Intra-assay | Pool A | 0.73 (4) | 0.13 (3) | 0.20 (2) | 0.6 | 2.5 | 0.9 |
| | Pool B | 3.01 (13) | 0.49 (4) | 1.00 (10) | 0.5 | 0.9 | 0.8 |
| Inter-assay | Pool A | 0.73 (30) | 0.13 (6) | 0.20 (9) | 5 | 5 | 4 |
| | Pool B | 2.98 (66) | 0.49 (10) | 1.02 (15) | 2.2 | 2.1 | 1.5 |

*Note*: The reproducibility of the assay was assessed by repeated measurements of two different glycated bovine bone hydrolysate sample pools with low (pool A) and high (pool B) crosslink concentrations. All results were measured by ten consecutive measurements. Results are expressed as mean (standard deviation).

**Table 3**
Quantitative analysis of crosslinks in various bone tissues.

| | *N* | Age (years) | PYD (mmol/mol coll) | DPD (mmol/mol coll) | PEN (mmol/mol coll) |
|---|---|---|---|---|---|
| Human trabecular lumbar bone | 30 | 80 ± 9 | 250 ± 67 | 104 ± 27 | 19 ± 8 |
| Bovine cortical bone | 5 | 10 | 368 ± 28 | 32 ± 2 | 1.8 ± 0.2 |
| Rat cortical bone | 10 | 3 months | 142 ± 36 | 190 ± 61 | <0.5 |
| Dog trabecular bone | 10 | 3 | 259 ± 31 | 39 ± 7 | 3.7 ± 0.9 |
| Sheep trabecular bone | 10 | 7 | 162 ± 36 | 13 ± 3 | 4.6 ± 1 |
| Chicken cortical bone | 10 | Foetal | 14.3 ± 1.6 | 5.7 ± 09 | N.D. |

*Note*: N.D.: non-detectable.

Crosslinks columns to avoid solid-phase variability packing density observed with home-made packed CF-1 cellulose columns. It was also reported that acetyl-pyridinoline and pyridoxine could be possible INT STD candidates [8,13]. However, these two molecules can present some disadvantages: acetyl-pyridinoline is destroyed by acid hydrolysis and pyridoxine is not retained via our SPE procedure (data not shown). Therefore, we tested the commercially available INT STD (BioRad) which is resistant to acid hydrolysis and retains well on the SPE column. This INT STD added before bone hydrolysis allows accurate quantification of the loss of crosslinks during sample pre-treatment. Finally, in our method, the molecules were separated on the SPE column and then on the reverse-phase column with a mobile phase containing acetonitrile instead of *n*-butanol because this solvent is easier to remove from the partition column and does not interfere with HPLC analysis. Therefore, the analytes that eluted from the SPE can be directly injected onto a C18 reverse-phase column avoiding the laborious concentration step. Moreover, the gradient containing acetonitrile showed greater performance and a better separation of the molecules and also gave specific retention time data for each analyte. PYD, DPD, INT STD and PEN were eluted around 11, 13, 23 and 35 min respectively.

### 3.3. Validation procedures

#### 3.3.1. Extraction recovery

The recovery rates calculated were 103 ± 3% for PYD, 100 ± 3% for DPD, 94 ± 4% for INT STD and 93 ± 4% for PEN. Pyridinium crosslinks were well recovered by using this new pre-treatment procedure. PEN is less retained than pyridinium crosslinks on SPE Chromabond® Crosslinks columns probably due to weaker affinity for the SPE matrix. However, PEN is accurately corrected by the INT STD (recovery tests) used in this procedure because these two molecules behave in a very similar way on the SPE.

#### 3.3.2. Linearity and calibration curve

The calibration curve of PYD, DPD, IS and PEN were described with the equation $y = 196{,}579x$, $y = 132{,}786x$, $y = 1{,}300{,}006x$ and $y = 237{,}679x$, respectively. The assay was linear over the validated amount range of 0–1 nmol for PYD, 0–0.5 nmol for DPD and PEN and over the validated relative amount range of 0–100% for INT STD. Correlation coefficients ($r^2$) of the calibration curves were 0.9998, 0.9998 and 0.9999 and 0.9999 for PYD, DPD, INT STD and PEN, respectively. The detection limit was as low as 0.2, 0.2 and 0.02 pmol for PYD, DPD and PEN respectively (data not shown).

#### 3.3.3. Reproducibility and precision

The intra- and inter-assay performance data are presented in Table 2. The intra-assay CVs were calculated from 10 consecutive analyses of the same pre-fractioned bone sample pools. The intra CVs for PYD, DPD and PEN were as low as 3%, respectively. The inter-assay CVs were calculated by HPLC injections of 10 different pre-treated bone sample pools. The inter-assay CVs for PYD, DPD and PEN were as low as 5%, respectively. The intra- and inter-assays variability tests demonstrated good reproducibility for the quantification of all of the collagen crosslinks.

### 3.4. Application

The developed assay was successfully applied to quantify collagen crosslinks in crude or demineralized bone hydrolysates. PYD, DPD and PEN were quantified in trabecular bone of human lumbar vertebrae, in trabecular bone of dog lumbar vertebrae and in cortical diaphysis femur of bovine and rat to validate this new method. Results of the crosslink contents are summarized in Table 3. From these results it is concluded that with our method, the collagen crosslinks contents estimated from crude bone hydrolysates are similar than those published after a demineralization step [14–20].

## 4. Conclusion

We developed and validated a rapid, simple, sensitive and specific assay for the quantification of collagen crosslinks in non-demineralized bone hydrolysates using HPLC. A simple clean-up procedure using SPE-treatment was found to yield sufficiently clean hydrolysates to allow the quantification of the molecules. The introduction of an INT STD in the procedure allowed to control and to improve the quantification of the crosslinks. This HPLC method avoided any necessary laborious and complicated sample pre-treatment steps and complex analyses.

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## References


[1] S. Viguet-Carrin, P. Garnero, P.D. Delmas, Osteoporos. Int. 17 (2006) 319.
[2] A.J. Bailey, R.G. Paul, L. Knott, Mech. Ageing Dev. 106 (1998) 1.
[3] L. Knott, A.J. Bailey, Bone 22 (1998) 181.
[4] D.R. Sell, R.H. Nagaraj, S.K. Grandhee, P. Odetti, A. Lapolla, J. Fogarty, V.M. Monnier, Diab. Metab Rev. 7 (1991) 239.
[5] D.R. Sell, V.M. Monnier, J. Biol. Chem. 264 (1989) 21597.
[6] D. Black, A. Duncan, S.P. Robins, Anal. Biochem. 169 (1988) 197.
[7] M. Takahashi, T. Ohishi, K. Aoshima, T. Inoue, K. Horiuchi, J. Liq. Chromatogr. 16 (1993) 1355.
[8] R.A. Bank, B. Beekman, N. Verzijl, J.A. de Roos, A.N. Sakkee, J.M. TeKoppele, J. Chromatogr. B Biomed. Sci. Appl. 703 (1997) 37.
[9] M. Takahashi, H. Hoshino, K. Kushida, T. Inoue, Anal. Biochem. 232 (1995) 158.
[10] S.K. Grandhee, V.M. Monnier, J. Biol. Chem. 266 (1991) 11649.
[11] U. Valcourt, B. Merle, E. Gineyts, S. Viguet-Carrin, P.D. Delmas, P. Garnero, J. Biol. Chem. 282 (2007) 5691.
[12] S. Viguet-Carrin, D. Farlay, Y. Bala, F. Munoz, M.L. Bouxsein, P.D. Delmas, Bone 42 (2008) 139.
[13] D.A. Pratt, Y. Daniloff, A. Duncan, S.P. Robins, Anal. Biochem. 207 (1992) 168.
[14] S. Viguet-Carrin, J.P. Roux, M.E. Arlot, Z. Merabet, D.J. Leeming, I. Byrjalsen, P.D. Delmas, M.L. Bouxsein, Bone 39 (2006) 1073.
[15] M.R. Allen, E. Gineyts, D.J. Leeming, D.B. Burr, P.D. Delmas, Osteoporos. Int. 19 (2008) 329.
[16] D.R. Eyre, T.J. Koob, K.P. Van Ness, Anal. Biochem. 137 (1984) 380.
[17] M. Saito, K. Marumo, K. Fujii, N. Ishioka, Anal. Biochem. 253 (1997) 26.
[18] M. Saito, S. Mori, T. Mashiba, S. Komatsubara, K. Marumo, Osteoporos. Int. 19 (2008) 1343.
[19] M. Saito, K. Fujii, Y. Mori, K. Marumo, Osteoporos. Int. 17 (2006) 1514.
[20] P. Garnero, O. Borel, E. Gineyts, F. Duboeuf, H. Solberg, M.L. Bouxsein, C. Christiansen, P.D. Delmas, Bone 38 (2006) 300.