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Chromatographic separation by RPLC-ESI-MS of all hydroxyproline isomers for the characterization of collagens from different sources

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ABSTRACT

During collagen biosynthesis, proline is post-translationally converted to hydroxyproline by specific enzymes. This amino acid, unique to collagen, plays a crucial role in stabilizing the collagen triple helix structure and could serve as an important biomarker for collagen content and quality analysis. Hydroxyproline has four isomers, depending on whether proline is hydroxylated at position 4 or 3 and on whether the *cis*- or *trans*- conformation is formed. Moreover, as extensive hydrolysis of collagen is required for its amino acid analysis, epimerization may also occur, although to a lesser extent, giving a total of eight possible isomers.

The aim of the present study was to develop a reversed-phase high-performance liquid chromatography-UV-mass spectrometry (RPLC-UV-MS) method for the separation and quantification of all eight hydroxyproline isomers. After the chiral derivatization of the hydroxyproline isomers with Nα-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA), to enable their UV detection, the derivatized diastereoisomers were separated by testing different C18 column technologies and morphologies and optimizing operative conditions such as the mobile phase composition (solvent, additives), elution mode, flow rate and temperature. Baseline resolution of all eight isomers was achieved on a HALO® ES-C18 reversed-phase column (150×1.5 mm, 2.7 μm, 160 Å) using isocratic elution and MS-compatible mobile phase.

The optimized method was validated for the quantification of hydroxyproline isomers and then applied to different collagen hydrolysates to gain insight and a deeper understanding of hydroxyproline abundances in different species (human, chicken) and sources (native, recombinant).

1. Introduction

Collagen, the most abundant protein in the human body, plays a crucial role in providing structural integrity to a wide range of tissues and organs [1–3] and has gained significant interest in biopharmaceutical fields, including drug delivery, skin regeneration and tissue engineering [4–7].

The structural stability and mechanical properties of collagen are heavily influenced by the presence of hydroxyproline (Hyp), a modified amino acid enzymatically introduced during collagen biosynthesis by prolyl-hydroxylases [8–10]. Specifically, the presence of 4-hydroxyproline residues along the amino acid sequence at the Y position of the repeated pattern $(Gly\text{-}X\text{-}Y)_n$, is fundamental for the stabilization and proper folding of the collagen triple helix [11,12]. Deficiencies or abnormalities in this process can result in the production of non-functional proteins, leading to severe biological consequences [13,14]. Limited information is available about 3-hydroxyproline, which is more likely located at position X within the repeated amino acid triplets. Despite its less frequent occurrence compared to 4-hydroxyproline, emerging evidence suggests that it may play an important yet under-explored role in maintaining collagen stability [15–18].

Hydroxyproline has two stereocenters, which involve the alpha-C atom and the hydroxylated carbon. Taking both 3- and 4-hydroxyproline into account, this results in the possibility of four stereoisomers, each with the potential to significantly impact collagen's biophysical properties and overall functionality. Additionally, under harsh sample treatment conditions, such as acid hydrolysis, epimerization can occur to a minor extent, leading to the formation of D-series amino acids [19].

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Therefore, the analysis of hydroxyproline should consider a total of eight stereoisomeric forms, whose identification and quantification are essential for comprehending collagen structure-function relationships and assessing the quality of collagen-based materials.

Collagen, as a biomaterial, can be extracted from natural animal sources [20] or obtained through recombinant protein production systems [21]. A great variation in the hydroxyproline content of collagen from various natural sources has been observed [22–24]. Within the context of recombinant collagen production which is still an ongoing challenge, the separation and quantification of all eight potential hydroxyproline isomers may bear great significance. This is particularly crucial, as to express a functional protein, the engineering of the host strain must encompass not only the gene for collagen itself but also the enzymes responsible for hydroxylation, namely prolyl hydroxylases. Hydroxyproline isomers can serve as valuable indicators of the quality, efficacy, and specificity of prolyl hydroxylases, and thus, of the overall success and reliability of the recombinant collagen production process.

Considering the laborious and time-consuming nature of existing collagen characterization methods [25], which limit accuracy, reproducibility, and sensitivity, it is not surprising that there is a persistent gap in knowledge regarding the understanding and quantification of hydroxyproline as well. Traditional methods for hydroxyproline analysis in collagen typically involve protein hydrolysis followed by pre-column derivatization and separation with LC. It is noteworthy that the majority of these methods date back to the last century [26–28]. In recent years, MS-based approaches have become more prominent, experiencing significant advancements in mapping this crucial post-translational modification (PTM) and investigating the potential occurrence of 4-hydroxyproline even at position X [29,30]. However, enzymatic treatment of collagen can yield peptides that are either excessively long or too short, which may hinder the efficacy of proteomics approaches and make quantification of hydroxyproline at the peptide level less accurate.

Currently, there is no comprehensive method available to separate all eight hydroxyproline isomers. Despite the work of Langrock et al. [31,32], who achieved the separation for six out of eight hydroxyprolines, challenges remain in achieving full resolution.

This work describes a novel RPLC-UV-MS method that serves as a robust analytical tool for the comprehensive separation and quantification of all eight hydroxyproline isomers that may be found in collagen. A mixture of the eight hydroxyproline standards (STDs) was subjected to derivatization with the chiral reagent Nα-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) to enable their separation by RP-LC and enhance UV detection. Chromatographic method development included column selection, explored the effect of mobile phase composition on retention and tested different elution modes to achieve efficient separation of the target analytes. Furthermore, the integration of MS enabled increased sensitivity in detection and thorough characterization of hydroxyproline isomers. The proposed method was subsequently validated and applied to collagen from different sources, including native and recombinant, to gain a better understanding of their hydroxyproline composition.

2. Materials and methods

2.1. Chemicals and reagents

(2S,4R)−4-hydroxypyrrolidine-2-carboxylic acid (*trans*-4-hydroxy-L-proline, *trans*-4-L-Hyp), proline, leucine, and isoleucine were purchased from Merck KGaA (Darmstadt, Germany). (2R,3S)−3-hydroxypyrrolidine-2-carboxylic acid hydrochloride (*cis*-3-hydroxy-D-proline, *cis*-3-D-Hyp) and (2S,3S)−3-hydroxypyrrolidine-2-carboxylic acid (*trans*-3-hydroxy-L-proline, *trans*-3-L-Hyp) were provided by Ambeed (Arlington Hts, IL, USA). (2R,3R)−3-hydroxypyrrolidine-2-carboxylic acid (*trans*-3-hydroxy-D-proline, *trans*-3-D-Hyp) and (2S,3R)−3-hydroxypyrrolidine-2-carboxylic acid (*cis*-3-hydroxy-L-proline, *cis*-3-L-Hyp) were obtained from Biosynth (Staad, Switzerland). (2R,4S)−4-hydroxypyrrolidine-2-carboxylic acid (*trans*-4-hydroxy-D-proline, *trans*-4-D-Hyp) and (2R,4R)−4-hydroxypyrrolidine-2-carboxylic acid (*cis*-4-hydroxy-D-proline, *cis*-4-D-Hyp) were from ThermoFisher Scientific (MA, USA), while (2S,4S)−4-hydroxypyrrolidine-2-carboxylic acid (*cis*-4-hydroxy-L-proline, *cis*-4-L-Hyp) from TCI Europe (Zwijndrecht, Belgium). Bovine serum albumin (BSA), collagen type II from chicken sternal cartilage, human collagen type IV, Nα-(2,4-dinitro-5-fluorophenyl)-L-valinamide (L-FDVA) and 6 M HCl in H_2O for amino acid analysis were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Water was obtained from a Direct-QTM Millipore system (Millipore, Billerica, MA, USA); acetonitrile (ACN) was purchased from PanReac AppliChem ITW Reagents (Cinisello Balsamo, Italy), while formic acid (FA) for mass spectrometry, trifluoroacetic acid (TFA) and $NaHCO_3$ were obtained from Merck KGaA (Darmstadt, Germany). Acetone was provided by Carlo Erba Reagents (Cornaredo, Italy). COL2A1 (human) native protein was obtained from Abnova (Taipei, Taiwan). Recombinant human procollagen type II as research raw sample was kindly provided by Gnosis by Lesaffre (Desio, Italy). Recombinant human procollagen type II has been obtained by using Pichia pastoris as host for the co-expression of gene hCOL2A1coding for human collagen α1 (II) chain, together with gene hαPH coding for human prolyl 4-hydroxylase and gene hPDI coding for human protein-disulfide isomerase.

2.2. Apparatus

Optimization of LC-UV method was conducted using an Agilent HPLC series 1200 (Santa Clara, CA, USA), equipped with an online mobile phase degasser, quaternary pump, autosampler, column thermostated compartment, diode array detector, and controlled by an Agilent ChemStation software (Rev. B.04.01).

HPLC-UV-ESI-MS analyses were carried out on a Dionex UltiMate 3000 HPLC (Thermo Scientific, San Jose, CA, USA) equipped with an autosampler, ternary pumps, UV detector, and coupled to a LTQ ion trap mass spectrometer with an electrospray ionization (ESI) ion source (Thermo Finnigan, San Jose, CA, USA). The system's control interface was X-Calibur software version 2.0.7.

2.3. L-FDVA derivatization of hydroxyproline standards

Hydroxyproline STDs were subjected to a derivatization reaction with L-FDVA using the established protocols outlined by Langrock et al. [32]. A 100 mM water stock solution was prepared for all hydroxyproline STDs, and then 10 μL of each was diluted with water at a 1:4 ratio. To each derivatization solution, 20 μL of 1 M sodium bicarbonate and 82.5 μL of 36.7 mM L-FDVA dissolved in acetone were added. The reaction mixtures were maintained at 40 °C under agitation for 90 min and then neutralized by adding 20 μL of 1 M HCl. To obtain a final 1 mM solution of each derivatized amino acid, 200 μL of ACN and 627.5 μL of water were added.

For method optimization, a combined derivatization mixture of all hydroxyproline isomers was also prepared by adding 10 μL of each hydroxyproline stock solution (10 mM) and maintaining the above ratios during the reaction. The mixture was then brought to a final volume of 4 mL with ACN and water in the adjusted proportions to obtain a stock solution of 2 mM (0.25 nmol/μL of each isomer).

2.4. HILIC-UV-ESI-MS control for the quantitative occurrence of the derivatization reaction

Underivatized 1 mM proline and hydroxyproline STDs were diluted 1:10 with ACN to obtain a final concentration of 0.1 mM. Similarly, the hydrolyzed and derivatized protein samples were treated as follows: the 50 μg/mL protein solution was diluted 1:10 with ACN.

For chromatographic analysis, a TSKgel® Amide-80 column (150×2 mm, 3 μm, 80 Å) was used and thermostated at 30 °C. The mobile phase consisted of 5 mM ammonium acetate, pH 5.5/ACN, 10:90 (solvent A)

and 5 mM ammonium acetate, pH 5.5/ACN, 40:60 (solvent B). Flow rate was set at 150 μL/min and elution involved a gradient from 5 % B to 60 % B over 28.5 min. Injection volume was 10 μL [32].

MS parameters included acquisition in positive ion mode with a scan range of 100–1000 *m/z* in full-scan mode. Source voltage was 4.5 kV, capillary voltage 31 V, sheath gas flow 27 and aux gas flow 11. Capillary temperature was set at 250 °C and tube lens voltage at 95 V.

2.5. Gas-phase hydrolysis and derivatization of protein samples

Individual amino acids were released from protein samples employing a simple gas-phase hydrolysis setup, as described by Langrock et al. [32]. Protein samples were prepared in water with 0.1 % FA at a concentration of 1 mg/mL. Next, 150 μL of the prepared samples were dried under a nitrogen flux in a glass insert. The insert was placed in a HPLC glass tube containing 300 μL of 6 M HCl specific for amino acid analysis, closed with its screw cap and subjected to hydrolysis at 110 °C for 24 h. Any remaining HCl in the glass insert was removed under nitrogen, and the dried hydrolysate was reconstituted with 15 μL of water.

For the derivatization reaction, 5 μL (50 μg of protein) were mixed with 16 μL of water and 8 μL of 1 M sodium bicarbonate. To the mixture, 32 μL of a 36.7 mM L-FDVA were then added and the reaction incubated as described in Section 2.3. The reaction was stopped by adding 8 μL of 1 M HCl and brought to a final volume of 1 mL with 200 μL ACN and 731 μL of water.

2.6. RPLC-UV-ESI-MS analysis

Method development for the separation of the eight total possible hydroxyproline isomers was carried out considering the following RP columns: Kinetex C18 (250×4.6 mm, 5 μm, 100 Å), YMC-Triart C18 (100×2.1 mm, 3 μm, 120 Å), Xterra MS C18 (250×2.1 mm, 5 μm, 125 Å), Kinetex C18 (100×2.1 mm, 5 μm, 100 Å).

Optimal separation was achieved using a HALO® ES-C18 column (150×1.5 mm, 2.7 μm, 160 Å) thermostated at 35 °C. The mobile phases used were water + 0.1 % FA (A) and ACN + 0.1 % FA (B) in a ratio 85:15. Isocratic elution was achieved at a constant flow rate of 200 μL/min, and the analytes were detected at 340 nm. The injection volume was set at 2 μL.

To ensure that all the species eluted before 80 min in the analysis of protein-derived samples, a linear increment from 15 to 60 % B in 20 min was introduced after 55 min of isocratic elution, followed by a 5-minute wash step at 90 % B.

The MS parameters used in positive ion mode were as follows: source voltage 4.6 kV, capillary voltage 44 V, sheath gas flow 18, auxiliary gas flow 15, capillary temperature 250 °C, tube lens voltage 75 V, and scan range 100–1000 *m/z* in full-scan mode.

2.7. RPLC-UV-ESI-MS method validation

Linearity was assessed using two distinct calibration ranges. The concentrations were established considering the theoretical concentration of total possible hydroxyproline in a 50 μg/mL solution of human procollagen type II based on primary sequence, estimated to be approximately 50 μM, and considered as 100 %. For *trans*-4-L-Hyp, a series of dilutions ranging from 6.25 to 75 μM (12.5 % to 150 %) was prepared. All the other isomers were evaluated at concentrations ranging from 0.1 to 5 μM each (0.2 %−10 %) by diluting the 2 mM STD mixture stock solution accordingly. Eight different calibration curves were thus generated.

Repeatability carried out on the following day, was determined by injecting three different preparations of the 100 % point for *trans*-4-L-Hyp curve (50 μM) and three of the 1 % point (0.5 μM) for the other Hyp isomers.

Recovery was calculated from a 50 μg/mL BSA protein sample that had been previously hydrolyzed and derivatized according to the procedure outlined in paragraph 2.5. BSA was used as a diluent to achieve a concentration of 50 μM for *trans*-4-L-hyp and 1.25 μM for the seven other isomers.

3. Results and discussion

The development and validation of a RPLC-UV-MS method, specifically designed to achieve the separation of all eight possible hydroxyproline isomers, were conducted after derivatization of the Hyp STDs mixture with L-FDVA. Method optimization focused on separating the four isomers of Hyp (4-Hyp and 3-Hyp, *cis* and *trans*) and their corresponding epimers that can be generated during the acidic hydrolysis of the protein. To achieve this, hyphenation with MS was employed to enhance sensitivity and ensure accurate identification. This is mandatory when processing hydrolyzed protein samples, where the presence of all derivatized amino acids can lead to co-elution, particularly problematic if considering only the UV trace. Therefore, the integration of MS with UV detection becomes essential to accurately identify target hydroxyprolines and avoid potential overestimation. After validation, the method was tested on different collagen hydrolysates. This investigation aimed to clarify differences in hydroxyproline composition across different species and production methods, including extraction from natural sources and recombinant expression.

3.1. Derivatization and control of hydroxyproline STD mixture

A well-established derivatization reaction involving hydroxyproline STDs with L-FDVA was chosen to generate diastereoisomers that can be separated on RP-LC, thereby enhancing UV detection sensitivity and allowing for more precise discrimination and quantification of Hyp isomers. This is particularly useful when dealing with potentially low-concentrated diastereoisomeric “impurities”. Arranged protocol from Langrock *et al.* [32] was then used in the preparation of the STD mixture.

Langrock assumed that the addition of a 3x molar excess of L-FDVA over amino acids would be sufficient for complete derivatization, thus enabling the quantitative determination of hydroxyproline. To confirm this assumption, HILIC-MS was used, as underivatized amino acids would elute at the front of the chromatogram in RPLC. The chromatographic conditions were adopted from Langrock’s work and the MS parameters were optimized as described in Section 2.4 of Materials and Methods. The analysis included underivatized proline STD and *trans*-4-L-Hyp STD, as well as their L-FDVA derivatives. The specific ion current was monitored at *m/z* 116 and *m/z* 132 for Pro-and 4-Hyp, respectively, and at *m/z* 396 and *m/z* 412 for their FDVA-derivatives. Underivatized Pro-had a retention time (RT) of 23.61 min, while *trans*-4-L-Hyp eluted at RT = 27.81 min (data not shown). In contrast, derivatized Pro-and Hyp eluted with the solvent front. To confirm the absence of residual non-derivatized amino acids, Pro-FDVA and Hyp-FDVA were injected. Notably, the extracted ion chromatograms (EICs) for non-derivatized amino acids resulted in no detectable signals

To reinforce this evidence, human collagen type II STD 1 mg/mL underwent acid hydrolysis and the same derivatization reaction, as detailed in Section 2.5 of Materials and Methods. In this instance as well, the search for *m/z* 116 and 132 signals in HILIC-MS analysis yielded no detectable peaks. The findings demonstrate that, even in complex samples and in the presence of other reacting amino acids, the applied method ensures satisfactory conversion of proline and hydroxyproline into L-FDVA derivatives. This is a prerequisite for an accurate detection and quantification of all hydroxyprolines in any biological samples.

3.2. RPLC-UV-MS method development

To achieve the optimal separation of the eight isomers, different factors that notably impact chromatographic separations were considered for appropriate method development. These factors included

mobile phase composition (solvent, additives), elution mode (isocratic, gradient), temperature, column dimensions and choice of stationary phase.

The initial column employed was a core-shell Kinetex C18 (250×4.6 mm) with a particle size of 5 μm and pore size of 100 Å. Initial separation attempts relied on existing literature evidence addressing the resolution of six out of eight Hyp isomers [31], and involved a mobile phase consisting of water and ACN, both added with FA, and shallow linear gradients. However, these conditions were not suitable for the baseline separation of all eight compounds, prompting further exploration of isocratic elution as well, to take advantage of the increased retention of the Hyp isomers within the stationary phase under isocratic elution.

In Table 1 is reported the elution order of the eight diastereoisomers in the isocratic analysis using 20 % ACN with FA. The correct identity for each peak was assigned by individual injection of the amino acid STDs.

Although the resolution values for adjacent peaks were successfully higher or close to the desired value of 1.5, in the cases of the lowest Rs the Hyp isomer couples resulted to be difficult to separate and were designated as critical pairs (CPs). The focus was on these CPs in order to further improve separation in method optimization attempts.

The impact of TFA, instead of FA, as mobile phase additive for the resolution of the CPs was first considered. As shown in Fig. 1 **(conditions B *vs* A)**, it was observed that the use of TFA increased the Rs of CP1 and, to a lesser extent, of CP2, but did not allow the optimal resolution value to be obtained for CP3. Due to the non-homogeneous behavior of the analytes concerning the ionic pairing agent added, the combined effect of the two additives was also investigated. Unfortunately, this approach did not lead to significant improvements, with a similar effect as the use of TFA alone (Fig. 1**, conditions C**).

Notably, a decrease in temperature (from 30 to 20 °C) revealed a negligible impact on resolution, except for CP1, increasing the retention times (Fig. 1**, conditions D**).

Methanol was also considered as an alternative to ACN, and due to its lower eluotropic strength, 30 % was used. A significant improvement in resolution was achieved for all pairs, but retention and thus analysis time (Fig. 1**, condition E**) nearly doubled. The prolonged elution time for the eight isomers and the associated increase in solvent consumption, when methanol was used, were considered unsatisfactory. The mobile phase containing ACN and FA was therefore confirmed as the most suitable.

To further improve resolution, alternative stationary phases were explored: ranging from 5 to 2.7 μm particle size, and including pure silica, hybrid silica and core-shell technologies (data not shown, see column details in Section 2.6). To address the need for high sensitivity and low solvent consumption, columns formats with I.D. ≤ 2.1 mm and length ≤ 150 mm were chosen.

Optimal chromatographic performance was achieved using a HALO® ES-C18 (150×1.5 mm, 2.7 μm, 160 Å); this sterically protected C18 bonded-phase exhibits very high column efficiency due to the narrow diffusion paths in the 0.5-micron thick, porous shell and the small overall particle size (2.7 μm). Under the best separation conditions (see Materials and Methods, paragraph 2.6) Rs values greater that 2.0 were achieved.

The method is fully compatible with ESI-MS and the ionization parameters have been optimized to ensure a stable spray current and MS signal. The specificity of MS detection adds an extra layer of selectivity to the chromatographic one, ensuring reliable quantification without interference from potential co-eluting species in complex protein-derived samples.

Representative chromatographic traces, illustrating the complete separation of the equimolar STD isomer mixture (50 μM each, corresponding to 100 pmol) are shown in Fig. 2, including the UV trace alongside the EIC at *m/z* 412 (Hyp-FDVA in positive ion mode). Chromatographic parameters for the eight hydroxyproline isomers are given in Table 2. Elution order was confirmed by injecting the STDs individually.

Table 1
Chromatographic data, obtained with Core-Shell Kinetex C18 (250×4.6 mm, 5 μm, 100 Å) thermostated at 30 °C using as mobile phase water + 0.1 % FA/ACN + 0.1 % FA (80:20) and flow rate of 1 mL/min, that allowed for the identification of critical pairs of isomers (CPs) difficult to resolve during method development. Detection was carried out at 340 nm and injection volume was 10 μL.

#	FDVA-Hyp	RT (min) ± SD	Resolution (Rs)	Critical pairs (CPs) of isomers
1	*trans*-4-D	21.383	/	
2	*trans*-4-L	22.137	1.39	CP1: #1-#2
3	*trans*-3-L	29.587	11.58	
4	*cis*-4-L	30.556	1.29	CP2: #3-#4
5	*cis*-4-D	33.641	3.82	
6	*trans*-3-D	36.445	3.22	
7	*cis*-3-L	37.788	1.46	CP3: #6-#7
8	*cis*-3-D	44.797	6.8	

3.3. RPLC-ESI-MS method validation

Given the intended application of the analytical method to collagen-based samples, validation was conducted on ESI-MS extracted ion chromatograms at *m/z* 412 (positive ion mode), acknowledging the potential for co-elution of different amino acids present in protein hydrolysates that could complicate the interpretation of UV traces alone and hinder UV quantitation.

Two distinct concentration ranges were examined: from 6.25 to 75 μM for *trans*-4-L-Hyp as it is the major compound found in collagen, accounting for over than 95 %, and from 0.1 to 5 μM for the lesser represented isomers, with the aim of achieving maximum sensitivity for any isomers that may be present at impurity levels. The 100 % point (50 μM) of the *trans*-4-L-Hyp curve was chosen on the basis of a theoretical calculation. It was assumed that 50 μg/mL of human STD collagen type II could generate a maximum of 0.045 μmol/mL of hydroxyproline, considering that approximately 127 proline residues in the α_1 chain are located in the required hydroxylation site (Y position of the Gly-X-Y_n repetitive amino acidic pattern). Eight different calibration curves were therefore generated, and a linear relationship was obtained from the peak areas of all the injected isomers within their respective concentration ranges: 6.25–75 μM (corresponding to 12.5–150 pmol) for *trans*-4-L-Hyp and 0.1–5 μM (0.2–10 pmol) for all other amino acids derivatives except for *cis*-3-D which ranged from 0.25 to 5 μM (0.5–10 pmol). These data represent a good improvement in terms of sensitivity compared to those previously reported [31,32]. The coefficient of determination (R^2) was >0.99 for all eight compounds in study and RSD on areas was less than 20 %. Calibration data for the examined Hyp isomers are detailed in Table 3. The data were generated from the average areas over three dilution series from a 2 mM standard mixture containing all the eight amino acid derivatives (250 μM each).

Repeatability was assessed on the following day by performing triplicate analyses at the 100 % point of the *trans*-4-L-Hyp curve and at the 1 % point for the remaining minor derivatives. The results showed RSDs consistently below 10 %, confirming the robustness and reliability of the analytical method (Table 4). Recovery was evaluated by supplementing a standard protein, bovine serum albumin, which lacks hydroxyproline, with 50 μM of *trans*-4-L-Hyp and 1.25 μM of other Hyp isomers. The results obtained fell within the acceptable limits, with recoveries ranging from 83 % to 117 % and RSD values below 20 %. Detailed recovery data are presented in Table 5 and the EICs at *m/z* 412 for both BSA and the spiked BSA are shown in Fig. 3A and B, respectively. Notably, the flat baseline in the BSA sample (Fig. 3A) confirms the absence of interferences at *m/z* 412 within the elution range of hydroxyprolines, highlighting the robustness of MS hyphenation. This underlines the reliability and specificity of the mass spectrometry detection method to accurately identify the target hydroxyproline in complex protein matrices.

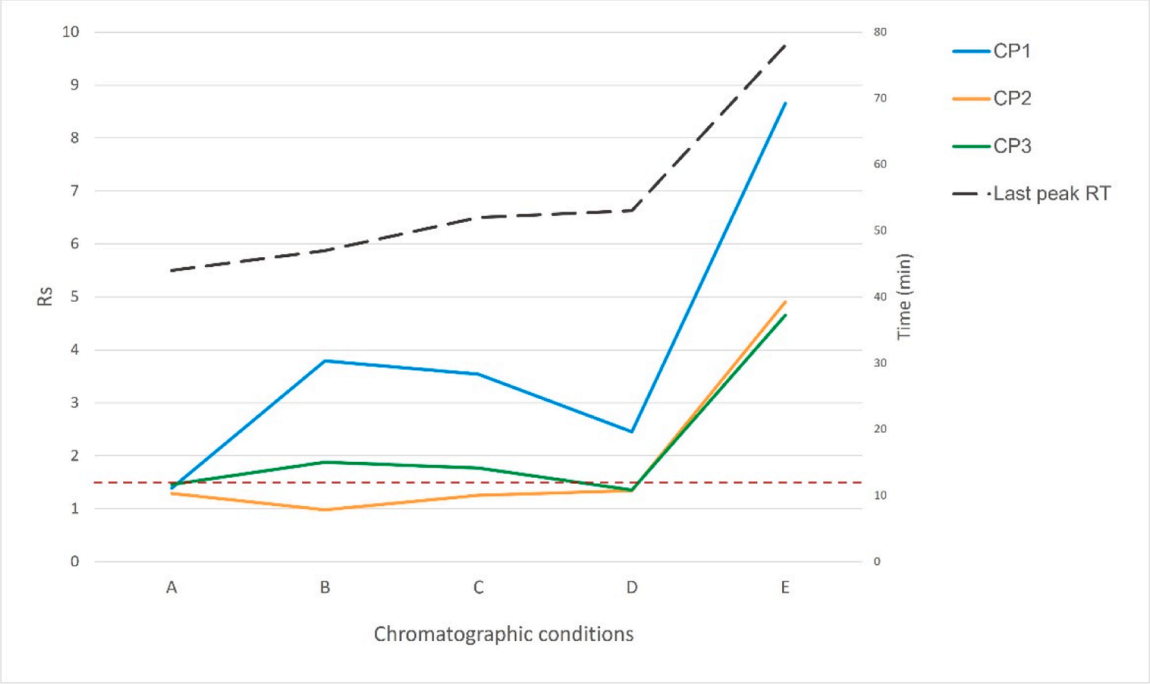

Fig. 1. Trend in CPs Rs values in the tested chromatographic conditions: A) ACN, 0.1 % FA, 30 °C; B) ACN, 0.1 % TFA, 30 °C; C) ACN, 0.1 % FA ± 0.05 % TFA, 30 °C; D) ACN, 0.1 % FA, 20 °C; E) MeOH, 0.1 % FA, 30 °C. Red dotted line refers to the Rs 1.5 threshold value.

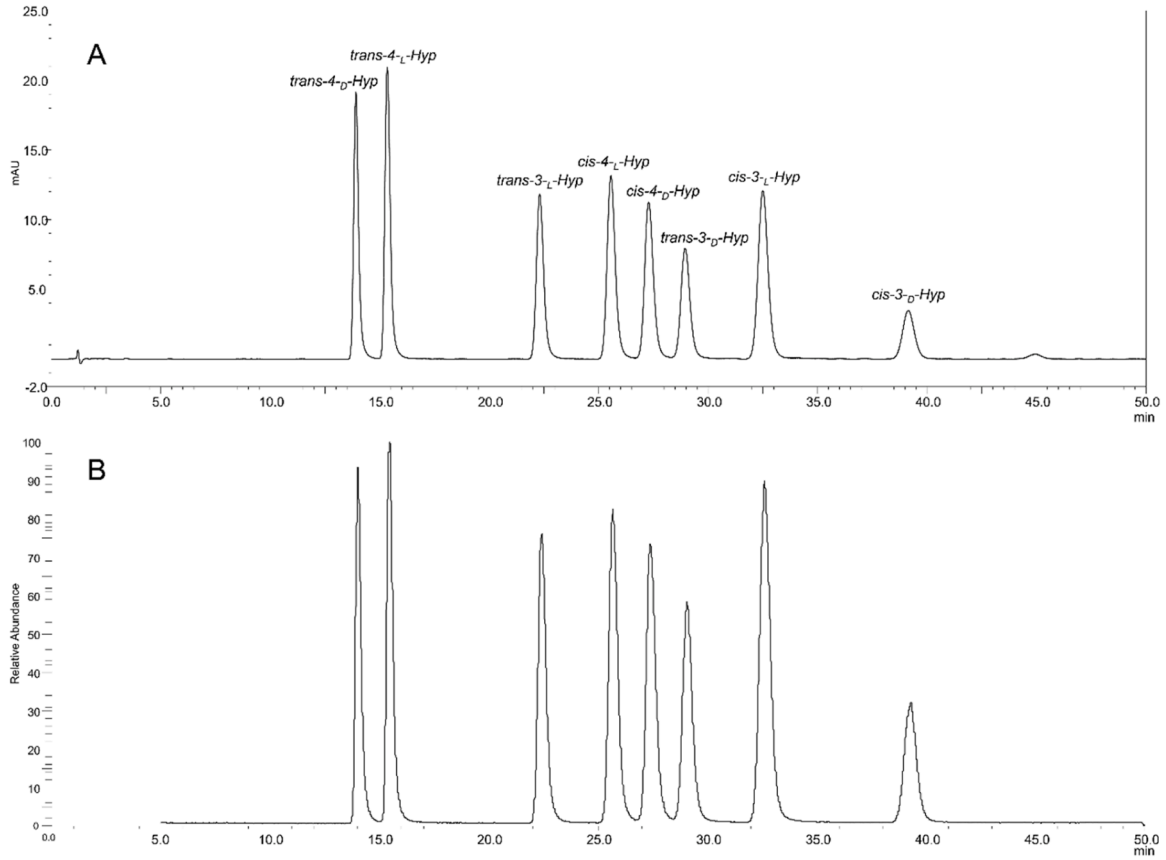

Fig. 2. RPLC separation of the L-FDVA derivatized eight hydroxyproline isomers (50 µM or 100 pmol each) carried out with a HALO® ES-C18 (150×1.5 mm, 2.7 µm, 160 Å). **A** UV trace of the STD mixture; **B** extracted ion chromatogram at *m/z* 412 (derivatized Hyp).

Table 2
Chromatographic data obtained with HALO® ES-C18 (150×1.5 mm, 2.7 μm, 160 Å) thermostated at 35 °C using as mobile phase water + 0.1 % FA/ACN + 0.1 % FA (85:15) and flow rate 200 μL/min. Detection was carried out at 340 nm and injection volume was 2 μL.

#	FDVA-Hyp	RT (min) ± SD	Resolution (Rs)
1	*trans*-4-D	13.88 ± 0.04	/
2	*trans*-4-L	15.32 ± 0.02	3.39
3	*trans*-3-L	22.23 ± 0.08	13.47
4	*cis*-4-L	25.42 ± 0.04	5.04
5	*cis*-4-D	27.15 ± 0.06	2.46
6	*trans*-3-D	28.82 ± 0.12	2.28
7	*cis*-3-L	32.37 ± 0.06	4.45
8	*cis*-3-D	39.05 ± 0.15	7.42

In the analysis of protein-derived samples a linear increment in ACN content was introduced after 50 min of isocratic elution to ensure that all species could be eluted before 80 min.

Under these conditions, the isobaric species isoleucine and leucine (*m/z* 412) elute distinctly from all hydroxyprolines at 67.97 ± 0.02 min and 68.26 ± 0.03 min, respectively, as can be seen in Fig. 3A and B. To confirm their identity and RTs, isoleucine STD and leucine STD were subjected to the same L-FDVA derivatization procedures and injected individually (data not shown).

3.4. Application to different collagen hydrolysates

The validated RPLC-UV-MS method was used to analyze collagen hydrolysates obtained from different species (human type II, chicken type II) and different sources, namely native and recombinant, in order to profile the specific Hyp isomer pattern. In addition, human collagen type IV, from the basement membrane was included in the study due to its higher content of *trans*-3-L-Hyp compared to type II, which is the articular collagen [9,33].

As can be seen in Table 6, both native chicken and human collagen predominantly contained over 90 % of *trans*-4-L-Hyp in addition to less than 5 % of its epimer, *cis*-4-D, which is formed by natural interconversion from *trans*-4-L-Hyp under harsh sample treatments, such as acid hydrolysis [31,32]. Moreover, the analyzed samples revealed the presence of *trans*-3-L-Hyp, the second most important isomer in collagen, which was below 2 % except for its double content in human collagen type IV. These findings are in agreement with literature data [9,33].

Interestingly, the content of *cis*-4-D-Hyp in recombinant procollagen was significantly higher (28 %), revealing a considerable difference between the native and the recombinant expression processes. This may be due to the different folding state of the alpha helix in the recombinant product compared to extracted collagen, which has a higher order fibrillar structure. Indeed, a variation in folding could increase the

Table 3
Calibration data obtained for all eight Hyp isomers using a linear regression equation ($y = mx + q$).

Linearity ($n = 3$)				
FDVA-Hyp	Intercept (q)	Slope (m)	Regression coefficient (R^2)	Concentration range for each compound (μM)
trans-4-D	283.09	4.850E+04	0.9970	0.1–5
trans-4-L	5927.00	8.248E+04	0.9865	6.25–75
trans-3-L	891.90	4.955E+04	0.9983	0.1–5
cis-4-L	−327.58	6.053E+04	0.9988	0.1–5
cis-4-D	−967.41	5.516E+04	0.9978	0.1–5
trans-3-D	113.55	4.299E+04	0.9966	0.1–5
cis-3-L	−644.40	7.481E+04	0.9977	0.1–5
cis-3-D	81.87	2.683E+04	0.9958	0.25–5

Table 4
Data on repeatability obtained on $n = 3$ different repetitions of the 100 % point of the calibration curve for *trans*-4-L-Hyp and 1 % point for all the other isomers, carried out on the following day.

Repeatability ($n = 3$)				
FDVA-Hyp	Concentration of each isomer (μM)	Mean area ± SD	RSD (%)	Recovered concentration (μM)
trans-4-D	0.50	27529 ± 2294	8.3	0.56
trans-4-L	50.0	4015679 ± 69935	1.7	48.7
trans-3-L	0.50	24831 ± 1745	7.0	0.48
cis-4-L	0.50	27007 ± 2306	8.5	0.45
cis-4-D	0.50	23554 ± 1164	4.9	0.44
trans-3-D	0.50	18781 ± 977	5.2	0.43
cis-3-L	0.50	33860 ± 1123	3.3	0.46
cis-3-D	0.50	13633 ± 727	5.3	0.51

Table 5
Recoveries of all eight Hyp isomers conducted by spiking 50 μg/mL of bovine serum albumin with 50 μM of *trans*-4-L-Hyp and 1.25 μM of other Hyps.

Recovery ($n = 3$)				
FDVA-Hyp	Concentration of each isomer added (μM)	Concentration found (μM)	RSD (%)	Recovery (%)
trans-4-D	1.25	1.16	17	93
trans-4-L	51.25	59.8	9	117
trans-3-L	1.25	1.04	10	83
cis-4-L	1.25	1.14	8	91
cis-4-D	1.25	1.21	6	97
trans-3-D	1.25	1.29	10	103
cis-3-L	1.25	1.32	9	106
cis-3-D	1.25	1.32	5	106

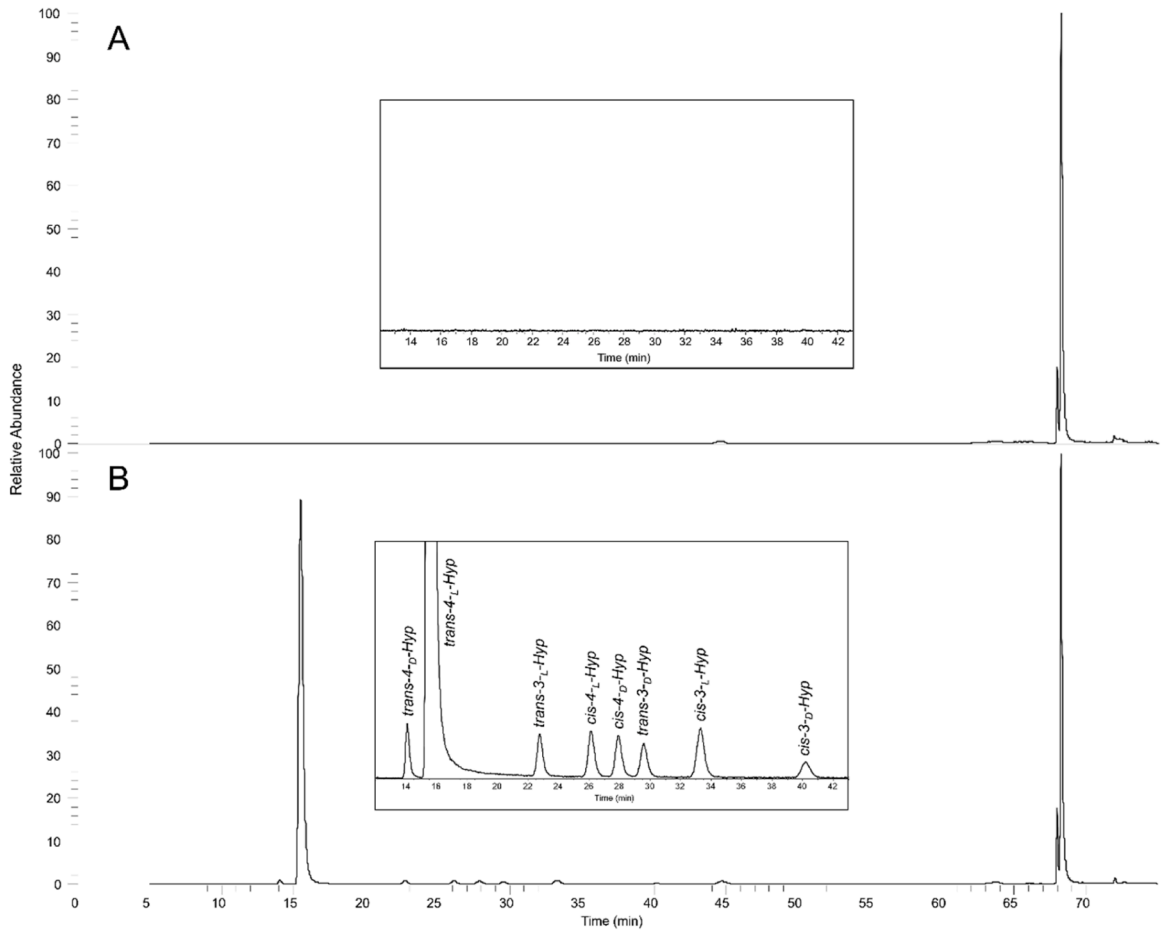

Fig. 3. EICs at *m/z* 412 of hydrolysed and derivatized (A) 50 μg/mL BSA and (B) 50 μg/mL BSA spiked with 50 μM *trans*-4-L-Hyp and 1.25 μM other Hyps.

Table 6
Percentage content of Hyp isomers found in collagen from different sources. The amount was calculated using the specific calibration curves for each isomer (Table 4).

	Hyp amount (%)		
Collagen	trans-4-L-Hyp	trans-3-L-Hyp	cis-4-D-Hyp
Human type II	95.6 ± 0.0	2.0 ± 0.1	2.4 ± 0.1
Human type IV	92.8 ± 1.3	3.9 ± 1.2	3.3 ± 0.1
Chicken type II	95.3 ± 0.4	1.4 ± 0.2	3.4 ± 0.6
Human type II, recombinant	72.0 ± 0.6	/	28.0 ± 0.6

accessibility of hydroxyproline during hydrolysis, resulting in a higher production of *cis*-4-D-Hyp. This finding suggests that the ratio of *trans*-4-L-Hyp to *cis*-4-D-Hyp, as well as that of the other epimers, may be a valuable marker of the folding status of the protein, highlighting the complex differences involved in recombinant protein production compared to biological systems. Despite this folding diversity, the substantial content of *trans*-4-L-Hyp in recombinant procollagen confirms successful hydroxylation by the engineered prolyl 4-hydroxylase (P4H) and underlines its specificity for 4-hydroxyproline, as further evidenced by the absence of *trans*-3-L-Hyp in the recombinant sample. The site-specificity of a recombinant enzyme, which is crucial for the post-translational modification of the collagen alpha chain, is another crucial point that must be properly assessed during the production process. Selective and sensitive separation and quantification of all the theoretical isomers is therefore a fundamental tool.

The total amount of Hyp was also considered as a marker for the origin of the collagen analyzed. The quantification of Hyp isomers in the three collagen samples revealed a total amount of 18.64 ng Hyp for human type II, 17.62 ng for human type IV, and 7.85 ng for chicken type II, corresponding to percentages (*w/w, ng Hyp/ng protein*) of 21 %, 20 % and 12 %, respectively. These differences are in agreement with the expected Hyp content based on the consensus sequences in the primary chain.

4. Conclusions

A simple and easily transferable isocratic RP-LC separation method for all eight hydroxyproline isomers based on a sterically protected C18 bonded phase (HALO® 160 Å ES-C18) has been optimized. The versatility of the method was further enhanced by its compatibility with ESI-MS, whose conditions were optimized to achieve a stable signal in positive ion mode, allowing the accurate detection of all Hyp isomers in the pmol range, representing a good improvement in sensitivity compared to previous works.

To ensure the reliability and robustness of the proposed method, an extensive validation process was carried out. Subsequently the method was tested on different collagen hydrolysates to improve the understanding of this still underexplored crucial PTM. The optimized RPLC-UV-MS method could be a useful tool not only for in-depth understanding of collagen hydroxylation, but also for quality control of collagen extracted from different natural sources or by recombinant protein production systems.

CRediT authorship contribution statement

Martina Lioi: Writing – original draft, Investigation, Formal

analysis. **Sara Tengattini:** Writing – review & editing, Data curation, Conceptualization. **Roberto Gotti:** Writing – review & editing, Methodology, Conceptualization. **Francesca Bagatin:** Investigation. **Stefano Galliani:** Investigation. **Gabriella Massolini:** Writing – review & editing, Supervision. **Simona Daly:** Writing – review & editing, Supervision, Funding acquisition. **Caterina Temporini:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Caterina Temporini reports financial support was provided by Italian Ministry of University and Research. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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