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# Analysis of hydroxyproline isomers and hydroxylysine by reversed-phase HPLC and mass spectrometry

Tobias Langrock, Natividad García-Villar, Ralf Hoffmann *

*Institute of Bioanalytical Chemistry, Center for Biotechnology and Biomedicine (BBZ), Faculty of Chemistry and Mineralogy, University of Leipzig, Deutscher Platz 5, 04103 Leipzig, Germany*

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**Abstract**

Collagens, the most abundant mammalian proteins, contain a high content of hydroxylated amino acids, such as, 3- and 4-*cis*-/*trans*-hydroxyproline (Hyp) and 5-hydroxylysine (Hyl). Whereas the global content of 4-Hyp was studied by amino acid analysis, no technique to determine all five hydroxyamino acids simultaneously in collagens has been reported. Here, we report the separation of all five hydroxyamino acids as well as two Hyp epimers from all other proteinogenic amino acids after derivatization with $N^2$-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (L-FDVA) by RPC-UV-ESI-MS. The general applicability of this method is shown for three Hyp-containing peptides as well as collagen type I.


*Keywords:* Electrospray; FDVA; Hydroxylation; Hyl; Hyp; RP-HPLC

## 1. Introduction

All multicellular organisms contain collagen, which is a family of fibrous proteins. They are the most abundant proteins in mammals constituting a quarter of the total protein content and form a major constituent of skin, bone, tendon, cartilage, blood vessels, and teeth. Collagens are present in nearly all organs and tissues and serve to hold the cells together in discrete units [1]. The most abundant collagen types are found in fibrils with a 67 nm axial period, formed by the axial staggering of collagen molecules. In addition, there are 14 "non-fibrillar" collagen types present in supramolecular forms such as networks and antiparallel arrays. All collagens form a specialized protein motif, the triple-helix, which is composed of three left-handed polyproline II (PPII)-like helical chains, wound around each other to form a tightly packed right-handed superhelix [2,3]. Only Gly residues with their small side chain can be accommodated without distortion in every third position to form a repeating sequence $(Gly–Xaa–Yaa)_n$. Xaa-positions are highly exposed, while residues in the Yaa-position are less accessible. In both Xaa- and Yaa-positions Pro is the most common residue, often oxidized to 4-hydroxy proline (4-Hyp) in the Yaa- and rarely to 3-Hyp in the Xaa-positions. Both Hyp and hydroxylated lysyl (Hyl) residues are known to play important roles in intra- and inter-molecular interactions and Hyp additionally stabilizes the triple-helix [4–6]. Recent work indicates that disturbed expression or enzymatic modifications of collagen are linked to some major diseases, such as osteoporosis and autoimmune diseases [7–9].

Although collagen sequences have been known for many years and the structural features are principally understood, the distribution of collagen types as well as the molecular distribution of posttranslational modifications has not been investigated in detail. This lack of research is related to the insolubility of many collagen types, their high molecular weight and the occurrence of repetitive sequences. Thus, separation of collagens by chromatographic or electrophoretic techniques is limited and sequence analysis by Edman-degradation or mass spectrometry is challenging. Furthermore, the unfavorable distribution of amino acids along the collagen sequence results in either very long or very short peptides after enzymatic or chemical cleavage complicating their analysis by current proteomics techniques. Therefore, the molecular distribution of the four Hyp-isomers present in collagens, that is, 4- and 3-Hyp in either *cis*- or *trans*-conformation, is still not well characterized. Especially *cis*-3-Hyp was excluded from most analyses due to the lack

* Corresponding author. Tel.: +49 341 9731331; fax: +49 341 9731339.
*E-mail address:* Hoffmann@chemie.uni-leipzig.de (R. Hoffmann).

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of analytical techniques to determine and quantify this imino acid.

A common approach to characterize collagens is amino acid analysis after acid hydrolysis in 6 mol/L aqueous hydrochloric acid. All four Hyp-isomers and Hyl are stable under these conditions; a significant degradation is not mentioned in the literature. Different methods based on gas or liquid chromatography have been described to analyze Hyp [10–13]. Most recent and sensitive techniques rely on precolumn derivatization, separation by reversed-phase HPLC followed by UV- or fluorescence-detection. Despite their relatively low sensitivity at the medium to low pmol-range, all these techniques are well suited, since collagens can be isolated in relatively large amounts. However, most techniques were originally developed for analysis of the regular 16 proteinogenic amino acids stable against acid hydrolysis. Whereas both 4-Hyp isomers are sometimes included, Hyl and 3-Hyp isomers are usually not. To our best knowledge, there is no report in the literature that describes separation and identification of Hyl and all four Hyp-isomers in the presence of proteinogenic amino acids. This is most likely attributed to the very similar retention behavior of the Hyp-isomers but also to the lack of appropriate amino acid standards. Only a specific derivatization of secondary amino acids with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride [14–16], differentiation by mass-spectrometry [17] or GC–MS [18] have been reported for the analysis of the four Hyp-isomers.

A further layer of complexity is added to the hydrolysates by partial epimerization of the Hyp-isomers. Although epimerization is only a side reaction at the 5% level, it forms 4-*cis*-D-Hyp from the dominant 4-*trans*-L-Hyp, which accounts for more than 90% of all Hyp-isomers in collagens. Whereas the diastereomers 4-*cis*-D-Hyp and 4-*trans*-L-Hyp could be separated with non-chiral reagents by RP-HPLC, 4-*cis*-D-Hyp cannot be separated from its enantiomer 4-*cis*-L-Hyp without derivatization with a chiral reagent or the use of a chiral stationary phase. Here, we describe a general approach to separate and quantify all four Hyp-isomers, *cis*-4-D-Hyp, *cis*-3-D-Hyp, Hyl, and the proteinogenic amino acids using a precolumn derivatization with an analogue of Marfey's reagent [19] based on $N^2$-(5-fluoro-2,4-dinitrophenyl)-L-valine amide (L-FDVA) instead of the originally described alanine amide [20]. By optimizing the acetonitrile gradient in the presence of formic acid as ion pair reagent, separation of all six studied Hyp-isomers was accomplished by RP-HPLC. This method was evaluated with collagen peptides containing either *trans*-4-Hyp, *trans*-3-Hyp or *cis*-4-Hyp and acid soluble collagen type I [21].

## 2. Experimental

All 20 proteinogenic L-amino acids, (2S, 4R)-4-hydroxypyrrolidine-2-carboxylic acid (*trans*-4-hydroxy-L-proline, *trans*-4-Hyp), (±)-*cis*-3-hydroxypyrrolidine-2-carboxylic acid (*cis*-3-hydroxy-D,L-proline, *cis*-3-Hyp), (2S, 3S)-3-hydroxypyrrolidine-2-carboxylic acid (*trans*-3-hydroxy-L-proline, *trans*-3-Hyp), (2R, 4R)-4-hydroxypyrrolidinecarboxylic acid (*cis*-4-hydroxy-L-proline, *cis*-4-Hyp), (2S, 4S)-4-hydroxypyrrolidinecarboxylic acid (*cis*-4-hydroxy-D-proline, *cis*-4-Hyp), and (2S, 5R)-2,6-diamino-5-hydroxycaproic acid dihydrochloride (5-hydroxy-L-lysine dihydrochloride, Hyl) and $N^2$-(5-fluoro-2,4-dinitrophenyl)-L-valine amide were purchased from Sigma–Aldrich–Fluka GmbH (Steinheim, Germany) at the highest purity available. All Fmoc-amino acids and reagents for peptide synthesis were from MultiSynTech GmbH (Witten, Germany).

### 2.1. Derivatization of amino acids

The studied α-amino and α-imino acids were derivatized with $N^2$-(5-fluoro-2,4-dinitrophenyl)-L-valine amide as originally described by Brückner and Keller-Hoehl [20]. All amino acids except tryptophan were dissolved in 20% aqueous acetonitrile at a concentration of 100 mmol/L. Due to its low solubility in 20% aqueous acetonitrile, Trp was dissolved at the same concentration in a 9:1 mixture of 25% ammonia and dimethylsulfoxide. Amino acids were individually derivatized by mixing 10 μL of the amino acid stock solution with 20 μL 1 mol/L $NaHCO_3$ and 40 μL water, followed by 82.5 μL L-FDVA reagent (36.7 mmol/L in acetone). The mixture was heated at 40 °C for 90 min. The reaction was stopped by addition of 20 μL 1 mol/L hydrochloric acid, the mixture was diluted with 200 μL acetonitrile and water to a final volume of 1 mL and the samples were stored at −20 °C in the freezer. Derivatization of all hydrolysates and mixtures of several amino acids, such as, all four Hyp isomers, was performed by the same procedure using the same reagent ratio.

### 2.2. Peptide synthesis

Peptides were synthesized on solid phase by Fmoc/*tert*-butyl-chemistry at a 25 μmol scale using diisopropyl carbodiimide/1-hydroxybenzotriazole activation on a multiple synthesizer (SYRO 2000), as described previously [22]. Solid support was a Wang resin yielding peptides with a C-terminal carboxyl group. Fmoc-*cis*-4-hydroxy-L-proline was synthesized according to a slightly modified procedure of Raillard et al. [23] and used for peptide synthesis without further purification. Both Fmoc-*cis*-4-Hyp and Fmoc-*trans*-4-Hyp were incorporated with unprotected hydroxyl groups.

### 2.3. Gas phase hydrolysis and derivatization

Acid soluble collagen I (Sigma type VIII) from rat tail, was obtained from Sigma–Aldrich–Fluka GmbH. Approximately 1 mg collagen was dissolved in 1 mL 50 mmol/L aqueous acetate buffer (pH 5.0). One hundred and fifty microliters of the solution were filled into small glass vial inlets (SUPELCO, Bellefonte, USA) and dried in vacuum. The glass vials were put in sealable plastic vials containing 150 μL 6 mol/L hydrochloric acid (hydrolysis grade). The vials were tightly closed and heated at 110 °C for 24 h. The hydrolysates were reconstituted with 15 μL water and one third of this solution derivatized with 32 μL L-FDVA solution diluted with 8 μL aqueous $NaHCO_3$ (1 mol/L) and 16 μL water. After heating at 40 °C for 90 min 8 μL 1 mol/L HCl were added, followed by 200 μL acetonitrile and water

to a final volume of 1 mL. Synthetic collagen peptides were hydrolyzed the same way, but the L-FDVA reagent amounts were reduced according to the total amino acid content (three-fold L-FDVA excess).

### 2.4. RPC and RPC-MS

Amino acid derivatives were separated on a HPLC Gold System (Beckman-Coulter, Krefeld, Germany) equipped with a 125NM gradient pump, a 168 NM photodiode array detector and a 508 auto sampler using an Aqua® C-18 column (150 mm × 2.0 mm, particle size 3 μm, pore size 125 Å) obtained from Phenomenex GmbH (Aschaffenburg, Germany). Alternatively, the HPLC-system was equipped with a Knauer UV-detector (Knauer GmbH, Berlin, Germany) and coupled on-line to a quadrupole time-of-flight hybrid mass spectrometer (QqTOF-MS, QSTAR Pulsar I, Applied Biosystems GmbH, Darmstadt, Germany) equipped with an ion-spray source (PE Sciex). The flow rate was 0.2 mL/min in both cases. The eluents were water containing 0.1% formic acid (eluent A) and acetonitrile containing 0.1% formic acid (eluent B). Two gradients were used both starting at 20% eluent B. The proteinogenic amino acid standard containing 19 L-amino acids (except cysteine) were separated by gradient 1 starting at 20% eluent B, which was linearly increased to 33% for 22 min and to 42% for 10 min. These conditions were hold for 10 min before the content of eluent B was increased to 70% within 10 min. Alternatively, a shallower acetonitrile gradient was applied to separate all four Hyp isomers (gradient 2). Starting also at 20% eluent B the percentage of eluent B was successively increased to 28% for 30 min, to 36% for 15 min, to 46.5% for 13 min, and to 65% for 10 min by linear increments. The derivatives were detected by their absorbance at 340 nm or by ESI-MS using a TOF-scan in positive ion-mode (5500 V), which was coupled on-line behind the UV-detector. The mass spectral data were recorded for *m/z* 300–800 to detect L-FDVA and all amino acid derivatives including the doubly derivatized Lys and Hyl.

## 3. Results

### 3.1. Separation and detection

L-FDVA derivatized amino acids allow separation and quantification of amino and imino acids as well as their enantiomers by RP-HPLC coupled on-line to ESI-MS [20]. The potential of this strategy to separate L- and D-enantiomers was important to analyze all L-Hyp-isomers present in collagens, that is, *trans*-4-Hyp, *cis*-4-Hyp, *trans*-3-Hyp, and *cis*-3-Hyp, as well as the dominant Hyp-epimers formed by racemisation of the α-C-atom during acid hydrolysis, that is, *cis*-4-D-Hyp and *cis*-3-D-Hyp. All six studied Hyp-isomers and -epimers were indeed well separated by a shallow acetonitrile gradient in the presence of 0.1% formic acid (Fig. 1). The *cis*-3-Hyp racemate was separated in two peaks at 32 and 35.5 min. Most likely *cis*-3-L-Hyp eluted before *cis*-3-D-Hyp (epimer of *trans*-3-L-Hyp), as FDVA derivatized L-amino acids typically elute earlier than the corresponding D-enantiomers [20]. However, the correct assignment will only be possible if enantiomerically pure compounds are available or *cis*-3-L-Hyp is identified in collagen. Most important all other proteinogenic amino acids as well as other byproducts eluted well outside this elution range. The Hyl derivative modified at both the α- and ε-amino group eluted in a single sharp peak at 68.7 min about two min before Lys.

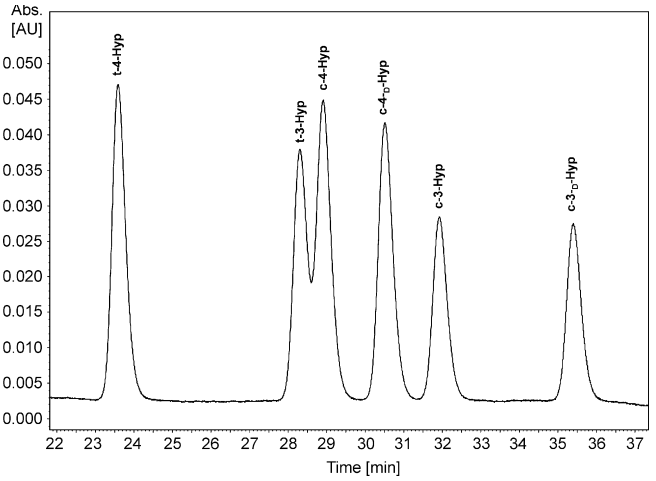


Fig. 1. A mixture of 200 pmol *trans*-4-Hyp, *cis*-4-Hyp, *trans*-3-Hyp, and *cis*-4-D-Hyp as well as 200 pmol of the racemic mixture of *cis*-3-Hyp was derivatized with $N^2$-(5-fluoro-2,4-dinitrophenyl)-L-valine amide. The derivatives were separated by RP-HPLC (C-18 Aqua column, length 150 mm, internal diameter 2 mm, particle size 3 μm) and detected at 340 nm. Both eluent A (water) and eluent B (acetonitrile) contained 0.1% formic acid. Elution was performed with linear increments from 20 to 28% eluent B for 30 min, to 36% eluent B for 15 min, to 46.5% eluent B for 13 min, and to 65% eluent B for 10 min.

In ESI-MS all derivatized amino and imino acids were stable under the applied ionization conditions, no significant in-source fragmentation was observed. Fragmentation was observed only after collision induced dissociation. However, the fragment ions and their intensities varied very much among the amino acids. Thus, it appeared impossible to use a single fragment ion or neutral loss for specific and sensitive detection of all amino acid derivatives in tandem mass spectrometry, which would improve the selectivity and the quantification. The same was true for negative ion-mode. All results presented below rely therefore on UV detection and a mass scan in positive ion-mode.

### 3.2. Analytical characteristics

The optimized HPLC gradient separated Hyl as well as all Hyp-isomers and -epimers from all other amino acids and further byproducts within 70 min. The peak areas showed a linear relationship to the injected amino acid amount within the studied range from 5 to 500 pmol, which was described by a regression equation $y = ax + b$ with $x$ being the concentration of the analyte (Table 1). The regression coefficients $R^2$ were typically better than 0.9997 except for *cis*-3-D-Hyp, which was 0.9984. This calculation based on three dilution series of a standard mixture containing all six Hyp-isomers and Hyl.

The standard deviation of five consecutive analyses on the same day was between 0.7% for Hyl and 2.9% for *cis*-3-D-Hyp

Table 1
Calibration data for the four studied L-Hyp-, both *cis*-D-Hyp isomers, and Hyl using a linear regression ($y = ax + b$)

| Amino acid | Intercept ($b$) | Slope ($a$) | Regression coefficient ($R^2$) |
|---|---|---|---|
| *trans*-4-Hyp | 8765.6 | 4444.9 | 0.9999 |
| *trans*-3-Hyp | 8839.6 | 3444.3 | 0.9997 |
| *cis*-4-Hyp | 6530.6 | 4047.7 | 0.9999 |
| *cis*-4-D-Hyp | 11039 | 4273.2 | 0.9998 |
| *cis*-3-Hyp | 12322 | 5548.1 | 0.9999 |
| *cis*-3-D-Hyp | −24470 | 6608.3 | 0.9984 |
| Hyl | −7585.1 | 7911.8 | 0.9997 |

Table 3
Recoveries of all six Hyp-isomers and Hyl, determined with Collagen Sigma type VIII using UV detection

| Amino acid | Mean value [pmol] | Amount addition [pmol] | Amount found [pmol] | Recovery [%] |
|---|---|---|---|---|
| *trans*-4-Hyp | 1841.2 | 1248.5 | 2915.3 | 86 |
| *trans*-3-Hyp | 32.7 | 109.3 | 130.6 | 90 |
| *cis*-4-Hyp | | 119.8 | 106.3 | 89 |
| *cis*-4-D-Hyp | 44.0 | 97.7 | 137.4 | 96 |
| *cis*-3-Hyp | 667.6[a] | 106.6 | 732.1 | 86 |
| *cis*-3-D-Hyp | 702.4[a] | 96.1 | 772.2 | 87 |
| Hyl | 359.4 | 138.8 | 491.4 | 95 |

[a] These values do probably not correspond to *cis*-3-Hyp but represent unidentified coeluting compounds, as the corresponding mass was not detected by mass spectrometry.

with a mean reproducibility of 1.4% (Table 2). The retention times shifted on average less than 9 s with a maximum of 12 s for *cis*-3-D-Hyp. The day-to-day precisions determined for three consecutive days using the average amino acid amount calculated from three analyses on each day were between 0.26 and 1.64% (average 1.06%). The recoveries of all five studied Hyp isomers and epimers as well as Hyl were in the range from 86 to 96%, which is in line with recovery rates obtained for other amino acids (Table 3).

No significant differences in the extinction coefficients and ionization degrees among the amino or imino acid derivatives were obtained, which was also valid for the negatively charged aspartic and glutamic acid (data not shown). The detection limits were about 5 pmol for UV absorption at 340 nm and 50 pmol in positive ion-mode ESI-TOF-MS. The relatively low sensitivity in ESI-MS might be attributed to the hampered ionization of amino-functionalized compounds in positive ion-mode. Alternatively, negative ion-mode ESI-MS was investigated due to the free carboxyl group common to all derivatives with respect to sensitivity and specificity as well as suppression of impurities including L-FDVA. As the sensitivity did not increase despite a decreased background noise, the negative ion-mode was not further pursued.

### 3.3. Sample analysis

Having established the separation of most proteinogenic amino acids as well as of all Hyp isomers including the *cis*-epimers, synthetic peptides (GPPGPPGPAGK) corresponding to a tryptic peptide of the collagen α1 (I) chain were used to test the established separation system. According to the sequence-specificity of collagen hydroxylases [24], three different peptides (GPPGPPGPAGK) were synthesized with Pro in position five being replaced by *trans*-3-Hyp, or Pro in position six by either *cis*-4- or *trans*-4-Hyp. Based on the retention times, peak areas and amino acid masses the peptides were quantitatively hydrolyzed (6 mol/L hydrochloric acid) without detectable degradation, isomerization or dehydration of either of the Hyp isomers after 24 and 48 h gas-phase hydrolysis using 75 pmol peptide. The chromatographic separation was also not influenced by the hydrolysate. The peak areas of the UV- and the MS-traces were almost identical among the three independently hydrolyzed peptides indicating the reliable identification and quantification of all amino acids, as shown in Fig. 2 for the *trans*-4-Hyp-containing peptide.

Finally, 50 μg (about 170 pmol) of a collagen type I sample (Sigma collagen type VIII) were hydrolyzed, derivatized and an aliquot of 1.7 pmol analyzed. The UV chromatogram was dominated from Gly and Pro, but displayed also intense peaks for *trans*-4-Hyp and weak peaks corresponding to *cis*-4-D-Hyp (data not shown). None of the 3-Hyp isomers was detected indicating that only few positions were accordingly modified. Thus, 10 times more derivatized sample (17 pmol) was injected for each collagen type to identify even hydroxylation of a single proline residue per collagen molecule by UV-absorption. The mass spectra were interpreted by extracting the signals at *m/z* 412 from the mass spectra corresponding to Hyp as well as the isobaric Leu and Ile (Fig. 3A). Two peaks were unambiguously assigned to *trans*-4-Hyp and *trans*-3-Hyp in the hydrolysates of collagen type I (Sigma type VIII, Fig. 3), whereas *cis*-4- and *cis*-

Table 2
Method validation with intraday and day-to-day precision for all six Hyp-isomers and -epimers as well as Hyl

| Amino acid | Intraday precision ($n = 5$) | | | Day-to-day precision ($n = 3$) | | |
|---|---|---|---|---|---|---|
| | Retention time ± S.D. [min] | Mean ± S.D. [pmol] | R.S.D. [%] | Retention time ± S.D. [min] | Mean ± S.D. [pmol] | R.S.D. [%] |
| *trans*-4-Hyp | 23.77 ± 0.12 | 298.4 ± 2.7 | 0.9 | 23.89 ± 0.1 | 297.43 ± 0.87 | 0.29 |
| *trans*-3-Hyp | 28.50 ± 0.14 | 213.0 ± 4.4 | 2.1 | 28.57 ± 0.08 | 209.86 ± 3.01 | 1.43 |
| *cis*-4-Hyp | 29.03 ± 0.15 | 323.8 ± 3.7 | 1.2 | 29.09 ± 0.09 | 324.22 ± 0.83 | 0.26 |
| *cis*-4-D-Hyp | 30.69 ± 0.17 | 243.2 ± 2.8 | 1.1 | 30.77 ± 0.1 | 241.45 ± 1.54 | 0.64 |
| *cis*-3-Hyp | 32.14 ± 0.18 | 125.2 ± 1.5 | 1.2 | 32.22 ± 0.12 | 124.31 ± 0.79 | 0.64 |
| *cis*-3-D-Hyp | 35.67 ± 0.21 | 113.7 ± 3.3 | 2.9 | 35.72 ± 0.11 | 112.27 ± 1.32 | 1.18 |
| Hyl | 68.69 ± 0.04 | 378.3 ± 2.6 | 0.7 | 68.89 ± 0.17 | 380.89 ± 6.25 | 1.64 |

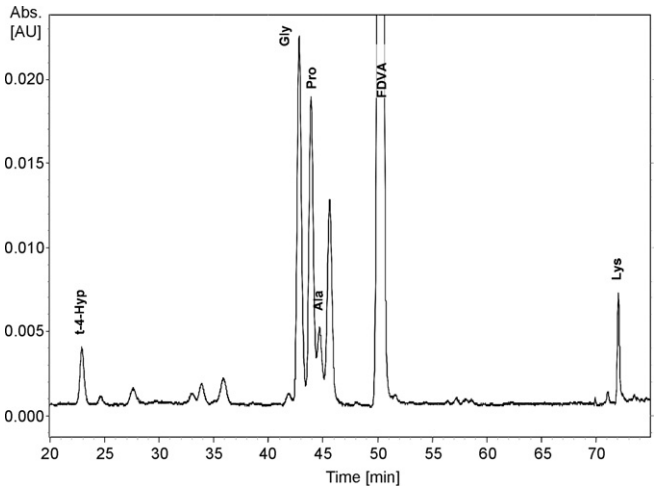


Fig. 2. UV trace of the GPPGP-$^{t}$4Hyp-GPAGK peptide hydrolysate derivatized with L-FDVA. For experimental details see Fig. 1.

Table 4
Contents of the detected Hyp-isomers and -epimers in collagen type I (Sigma type VIII) hydrolysate (6 mol/L HCl, 24 h, 108 °C) using UV-detection (340 nm) and MS-detection in positive ion-mode

| Imino acid | Collagen type I (Sigma type VIII) | | | | Average |
|---|---|---|---|---|---|
| | UV-detection | | MS-detection | | |
| | Height [%] | Area [%] | Height [%] | Area [%] | |
| *trans*-4-Hyp | 95.6 | 95.5 | 94.4 | 95.1 | 95.1 |
| *trans*-3-Hyp | 1.7 | 1.8 | 1.8 | 1.5 | 1.7 |
| *cis*-4-D-Hyp | 2.7 | 2.7 | 3.8 | 3.4 | 3.2 |

The average was calculated from the peak height and the area of both UV- and MS-detection.

3-Hyp were not detected at all. Integration of the peak areas in the UV chromatograms and the MS-traces yielded 95.1% *trans*-4-Hyp and 1.7% *trans*-3-Hyp in collagen I (Fig. 3; Table 4). The remaining 3.2% were *cis*-4-D-Hyp formed from *trans*-4-Hyp. For the UV-trace it might be favorable to quantify Hyp by the peak heights due to the bad separation of close eluting unknown byproducts present in the collagen hydrolysates (Fig. 3B).

The analysis of lysine and hydroxylysine relied on the doubly derivatized compounds using mass extraction and UV-absorption. The Hyl to Lys ratios were 1 to 2.1. UV-quantification of Hyl and Lys was possible, as both derivatives were baseline separated from each other and all other amino acids.

## 4. Discussion

An important side reaction of amino acid hydrolysis is racemization of the α-C-atom, which yields up to 10% D-enantiomer for some amino acids [25]. In the case of Hyp, the corresponding epimers are formed, such as *cis*-3-D-Hyp from *trans*-3-L-Hyp and *cis*-4-D-Hyp from *trans*-4-L-Hyp. As *trans*-4-Hyp is present in collagens at a relatively high content, significant amounts of *cis*-4-D-Hyp are formed during acid hydrolysis. These epimers have to be separated from all other Hyp-isomers for reliable quantification by UV or MS. For this reason we had chosen the chiral derivatization reagent L-FDVA. However, the originally in the literature described eluent system with trifluoroacetic acid (TFA) separated only the *cis*-3-Hyp enantiomers but not the *cis*-4-Hyp enantiomers (data not shown). Neither shallower gradients, nor other stationary phases nor methanol gradients allowed separation of the two *cis*-4-Hyp enantiomers. Only replacement of TFA as ion pair reagent by formic acid was successful to separate all four L-Hyp and both *cis*-D-Hyp isomers from each other and all other proteinogenic amino acids (Fig. 1). As for both 3- and 4-Hyp isomers the *trans* configuration dominates in collagens, only the corresponding *cis*-D-epimers have to be considered. Any *trans*-3- or *trans*-4-D-Hyp formed by the hydrolysis conditions would be well below the detection limit and should therefore not interfere with quantification of the targeted derivatives. The replacement of TFA by formic acid has also advantages for the ESI-MS coupling, as TFA clusters contaminating the mass spectrometer are omitted, the ionization is more effective, and the spray conditions are more stable.

A major challenge of Hyp analyses in collagen hydrolysates besides separation of all Hyp-isomers and -epimers are their large concentration differences spanning two orders of magnitude. Gly and Pro are the dominating amino acids with a content of 27% and 19% for the α1-chain and 28% and 17% for the α2-chain in human collagen type I. About 10% Pro residues are hydroxylated in collagens, mostly in the *trans*-4-position and less than 2% in the *trans*-3-position. The corresponding *cis*-isomers are again about 10 times less likely. Thus, these imino acids have to be quantified in the presence of 100–1000 times higher concentrated amino acids. At this very level low UV-detection might be questioned, as derivatized D-amino acids formed during the acid hydrolysis as well as not completely hydrolyzed peptides, especially polar di- and tripeptides, might coelute with some Hyp-isomers. Thus, MS quantification was validated for Hyp-isomers just above the detection limit. Based on Hyp-standards a linear response was obtained from 100 to 500 pmol ($R^2 \geq 0.994$), which could be further extended by a non-linear regression. It should be noted that the used time-of-flight mass spectrometer has a low linear range, but that quantification was very reliable, especially as not the absolute amount of each amino acid but only the relative content of three Hyp-isomers and -epimers was studied in this concentration range.

Interestingly, the relative contents of all three identified Hyp-isomers and -epimers were very similar in the UV- and MS-traces confirming that the MS signal intensities can be used to quantify Hyp. This strongly suggests that no further FDVA-derived contaminants coeluted with either Hyp-isomer. The intensity of the *cis*-4-D-Hyp epimer was about 3% relative to *trans*-4-L-Hyp, which was within the expected range for acid hydrolysates. As the *cis*-4-D-Hyp is formed from *trans*-4-L-Hyp during hydrolysis, the *trans*-4-L-content of the collagens has to be corrected accordingly. Thus, the data above show that hydroxylation of proline in collagens forms almost 98% *trans*-4-Hyp in Sigma type VIII collagen.

The lysine and hydroxylysine content of the collagen types was determined by the peak areas of the doubly derivatized compounds. Besides these two peaks, four signals corresponding most likely to Lys and Hyl modified either in α- or ε-position

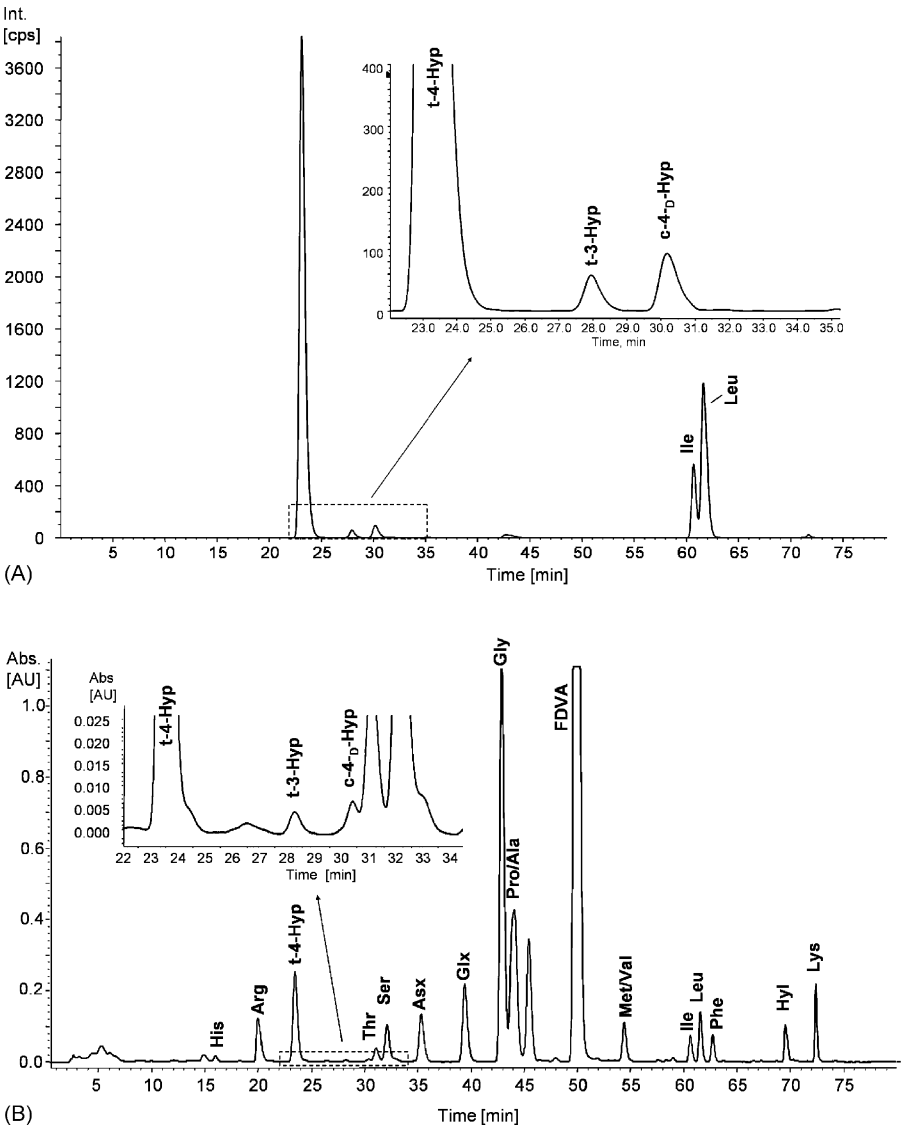


Fig. 3. Separation of a L-FDVA derivatized collagen I hydrolysate (17 pmol) on a C-18 Aqua column. The separation conditions were the same as described in Fig. 1. The upper panel displays the extracted masses corresponding to the isobaric derivatives of Hyp, Leu, and Ile. The ESI-MS data were recorded on a QSTAR Pulsar I mass spectrometer equipped with an ion-spray-source (5500 V). The lower panel shows the corresponding UV trace.

were detected at a low intensity by ESI-MS. The very low signal intensities of the mono-derivatized compounds in UV indicated that quantification can rely only on the intensities of doubly derivatized Lys and Hyl without significant errors. The stronger signal intensities of the mono derivatized compounds in ESI-MS are most likely attributed to their free amino groups that should significantly increase the ionization degree in ESI-MS relative to all fully derivatized amino acids. Thus, both Lys and Hyl were quantified by integration of the peak areas of the doubly derivatized compounds.

In conclusion, the described method allows a fast, reliable and sensitive quantification for all L-Hyp isomers and Hyl present in collagens using UV detection at 340 nm. However, the very low modification level for some amino acids limits UV detection, as other UV active impurities may coelute. Thus, the data should be confirmed by mass spectrometry, at least for low signal intensities. Whereas the TOF instrument used in this study is not an ideal tool to quantify the amino acid derivatives, quadrupole or ion trap mass analyzers equipped with an ESI source should allow quantification down to the 5 pmol range with a dynamic range spanning more than three orders of magnitude.

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## References

[1] T. Philajaniemi, R. Myllyla, K.I. Kivirikko, J. Hepatol. 12 (1991) S2.
[2] A. Rich, F.H. Crick, J. Mol. Biol. 3 (1961) 483.

[3] G.N. Ramachandran, Treatise on Collagen, Academic Publishers, New York, 1964.
[4] J. Bella, M. Eaton, B. Brodsky, H.M. Berman, Science 266 (1994) 75.
[5] D.A. Slatter, C.A. Miles, A.J. Bailey, J. Mol. Biol. 329 (2003) 175.
[6] C. Kannicht (Ed.), Posttranslational Modifications of Proteins: Tools for Functional Proteomics, Humana Press Inc., 2002.
[7] K.M. Jorden, C. Cooper, Best Pract. Res. Clin. Rheumatol. 16 (2002) 795.
[8] J. Broddefalk, J. Bäcklund, F. Almqvist, M. Johannson, J. Kihlberg, J. Am. Chem. Soc. 120 (1998) 7676.
[9] R. Holmdahl, M. Andersson, T. Goldschmidt, K. Gustafsson, L. Jansson, J.A. Mo, Immunol. Rev. 118 (1990) 193.
[10] M. Delport, S. Mass, S.W. van der Merwe, J.B. Laurens, J. Chromatogr. B 804 (2004) 345.
[11] Y. Tsuruta, H. Inoue, Anal. Biochem. 265 (1998) 15.
[12] H. Inoue, H. Iguchi, A. Kono, Y. Tsuruta, J. Chromatogr. B 724 (1998) 221.
[13] E. Kindt, K. Gueneva-Boucheva, M.D. Rekhter, J. Humphries, H. Hallak, J. Pharm. Biomed. Anal. 33 (2003) 1081.
[14] G. Bellon, R. Berg, F. Chastang, A. Malgras, J.P. Borel, Anal. Biochem. 137 (1983) 151.
[15] W.J. Lindblad, R.F. Diegelmann, Anal. Biochem. 138 (1984) 390.
[16] A. Bisker, V. Pailler, A. Randoux, J.P. Borel, Anal. Biochem. 122 (1982) 52.
[17] D.B. Kassel, K. Biemann, Anal. Chem. 62 (1990) 1691.
[18] T. Erbe, H. Brückner, Z. Lebensm. Unters. Forsch. 208 (1999) 424.
[19] P. Marfey, Carlsberg Res. Comm. 49 (1984) 591.
[20] H. Brückner, C. Keller-Hoehl, Chromatographia 40 (1990) 621.
[21] R. Bornstein, W. Traub, Proteins 4 (1979) 411.
[22] R. Pipkorn, C. Boenke, M. Gehrke, R. Hoffmann, J. Pept. Res. 59 (2002) 105.
[23] S.P. Raillard, A.D. Mann, T.A. Baer, Org. Prep. Proced. Int. 30 (2) (1998) 183.
[24] K.I. Kivirikko, T. Pihlajaniemi, Adv. Enzymol. Relat. Areas Mol. Biol. 72 (1998) 325.
[25] M. Weiss, M. Manneberg, J. Juranville, H. Lahm, M. Fountoulakis, J. Chromatogr. A 795 (1998) 263.