RPLC-MS analytical method for the separation of all hydroxyproline isomers and application to different collagen hydrolysates

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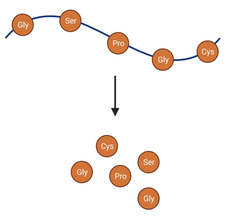
P-BPHA23

Best Poster Award

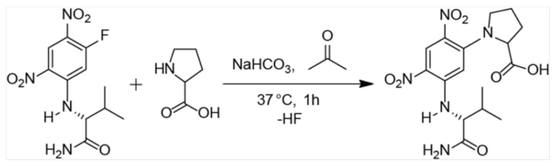
WORKFLOW

GAS-PHASE HYDROLYSIS



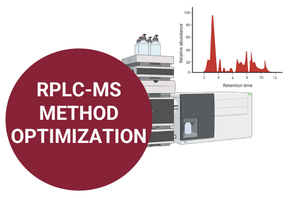
Protein hydrolysis with HCl 6M for 24 hours at 110 °C

DERIVATIZATION[3]



Amino acid reaction with 3x molar excess of the chiral derivatization agent L-FDVA

RPLC-MS ANALYSIS



Method development with Hyp STDs and application to protein hydrolysates

3. H. Bruckner, C. Keller-Hoehl, Chromatographia 30, 621-629 (1990)

METHOD DEVELOPMENT

We dedicated ourselves to finding a method that could effectively separate and quantify all potential isomers of hydroxyproline in collagen. Several chromatographic columns were evaluated to determine their ability to resolve selected critical pairs of isomers, aiming to identify the most suitable approach.

TESTED COLUMNS	
Quattro S C18 3 µm 120 A (100 x 2.1 mm)	Silica
YMC-Triart C18 3 µm 120 A (100 x 2.1 mm)	Hybrid silica
Xterra MS C18 5 µm (250 x 2.1 mm)	Hybrid silica
Kinetex C18 5 µm 100 A (100 x 2.1 mm)	Core-Shell
Kinetex C18 5 µm 100 A (250 x 4.6 mm)	Core-Shell
Halo ES-C18 2.7 µm 160A (150 x 1.5 mm)	Core-Shell

PAIRS OF CRITICAL PEAKS IDENTIFIED (CPs)	
CP_1 = #1 - #2	T4D-T4L
CP_2 = #3 - #4	T3L-C4L
CP_3 = #4 - #5	C4L-C4D
CP_4 = #6 - #7	T3D-C3L

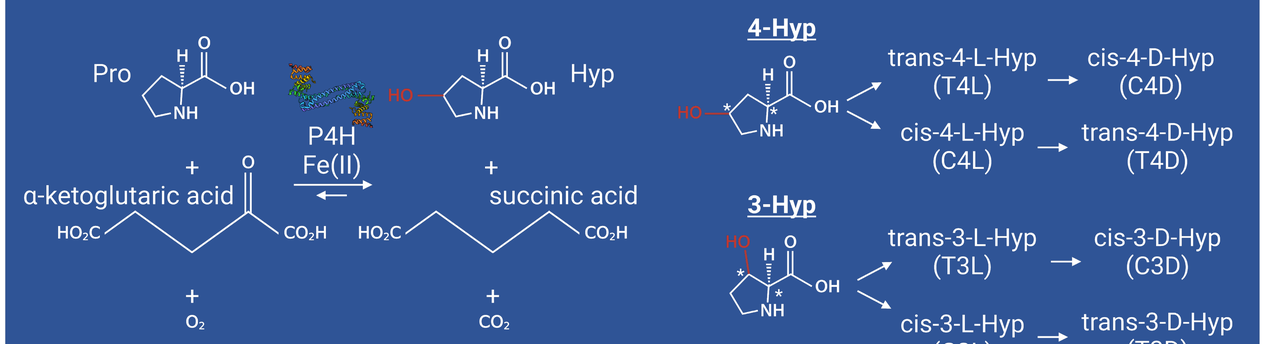
Different chromatographic conditions were explored using a Kinetex C18 5 µm 100 A (250 x 4.6 mm). Despite methanol yielded high resolutions for all CPs, the analysis time and solvent consumption were considered unsatisfactory (Fig.1). Therefore, focusing on the use of acetonitrile, and formic acid as mobile phase additive, excellent results were attained using a Halo ES-C18 2.7 µm 160 A (150 x 1.5 mm), which was ultimately chosen for method validation (Fig.2).

PRE-VALIDATION

The hydroxyproline isomers calibration curves were constructed covering different concentration levels (0.15 mM, 0.08 mM, 0.02 mM, 0.0015 mM). A semi-validation was performed and the data are shown in table below.

						INTRA-DAY PRECISION (RSD%)	
#	ISOMER	Linearity	R^2	LOD (mM)	LOQ (mM)	0.15 mM	0.0015 mM
1	T4D	y = 122.14x + 0.0311	0.999	0.011	0.033	1.9	0.7
2	T4L	y = 144.7x – 0.006	0.999	0.011	0.034	2.1	0.9
3	T3L	y = 111.24x – 0.0214	0.999	0.012	0.035	0.9	1.9
4	C4L	y = 137.81x – 0.0492	0.999	0.012	0.037	1.9	4.8
5	C4D	y = 121.61x – 0.0528	0.999	0.012	0.037	1.2	2.0
6	T3D	y = 90.222x – 0.0251	0.999	0.012	0.035	1.5	2.9
7	C3L	y = 152.44x + 0.0057	0.999	0.011	0.034	2.0	4.4
8	C3D	y = 49.536x – 0.001	0.999	0.011	0.032	1.9	6.1

In collagen biosynthesis, the enzyme prolyl 4-hydroxylase (P4H) plays a crucial role by inserting 4-hydroxyproline into newly synthesized polypeptide chains. This essential post-translational modification ensures proper folding of the collagen triple helix. Inhibition of this process leads to non-functional protein secretion, resulting in impaired collagen formation. P4H catalyzes the addition of a hydroxyl group to proline, which must be present in the specific pattern -X-Pro-Gly- along collagen chains[1]. Since hydroxyproline has two stereocenters including the α-C atom and the hydroxylated C, four stereoisomers can exist both for 3-hydroxyproline and 4-hydroxyproline. Additionally, during acid hydrolysis, racemization can occur to a lesser extent, leading to the formation of α-D-amino acids[2].



1. T. Pihlajaniemi, R. Myllylä, K.I Kivirikko, J. Hepatol. 13, S2-S7 (1991)
2. T. Langrock, N. García-Villar, R. Hoffmann, J. Chromatograph. B 847, 282-288 (2007)

METHOD SCOUTING

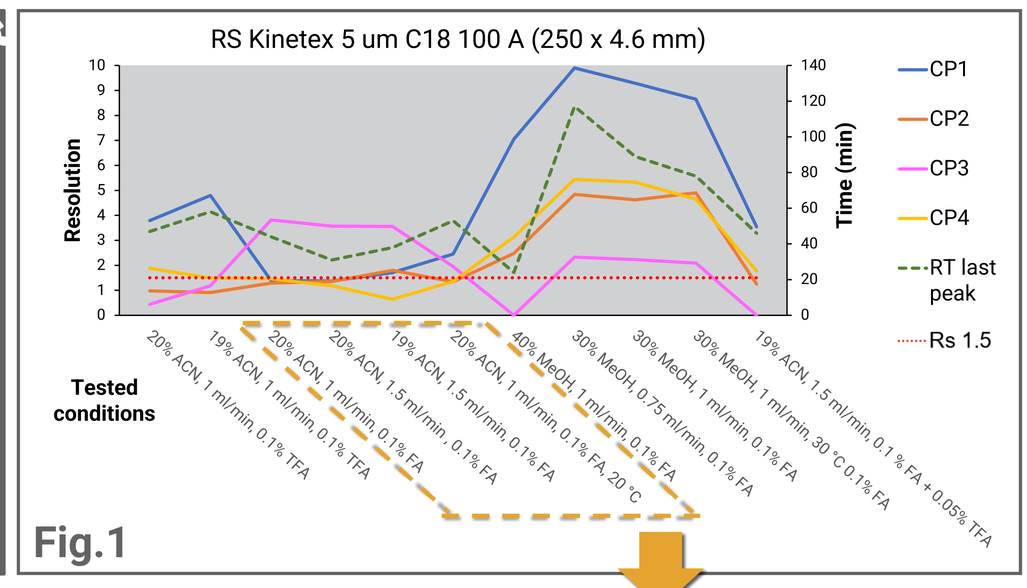


Fig.1

OUTCOMES

#	ISOMER	Rs
1	T4D	
2	T4L	4.06
3	T3L	14.75
4	C4L	5.47
5	C4D	2.46
6	T3D	2.54
7	C3L	4.62
8	C3D	7.28

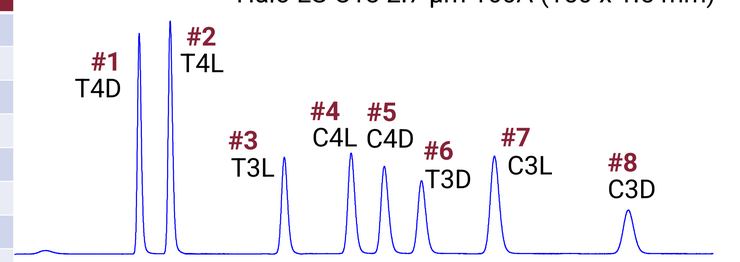


Fig.2

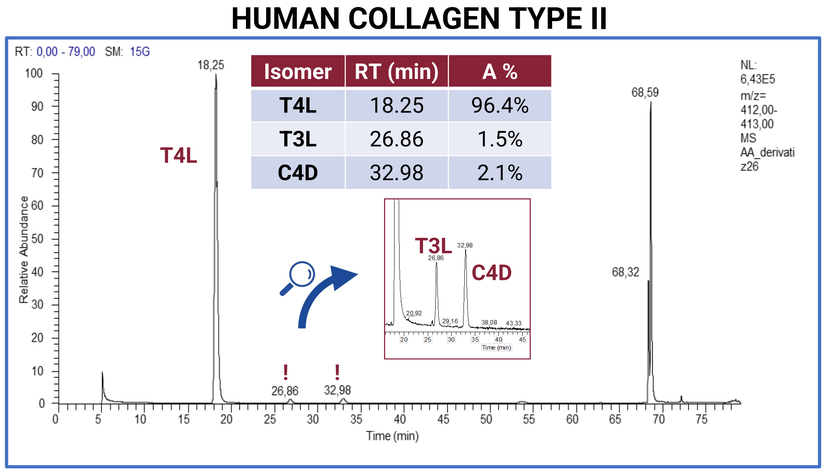
RP-LC PARAMETERS mobile phases: A) H2O + 0.1% FA, B) ACN + 0.1% FA; flow: 0.2 ml/min; elution: isocratic; inj volume: 2 µL; UV wavelength: 347 nm. The chromatographic conditions are MS compatible.

We thank CPS Analitica and Advanced Materials Technology for having kindly provided us with the «Halo ES-C18 2.7 µm 160A (150 x 1.5 mm)» column, we sincerely appreciate your support.

APPLICATION TO DIFFERENT COLLAGEN HYDROLYSATES

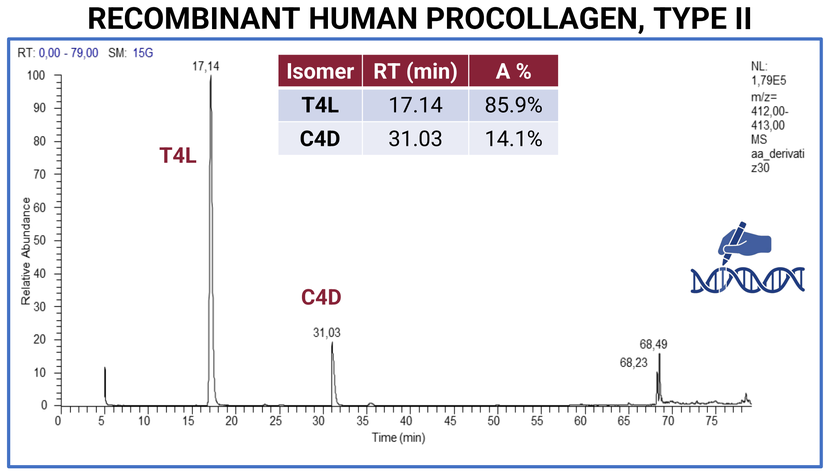
In the field of recombinant collagen production, which continues to be a challenge, the separation and quantification of all eight possible hydroxyproline isomers may hold great significance. These isomers serve as valuable indicators of quality, efficacy, and specificity of P4H and, therefore, of the overall success and reliability of the recombinant collagen production process. Our RPLC-UV method, coupled to MS, was tested on different collagen samples to gain insight on their hydroxyproline composition. XICs were at m/z = 412 corresponding to derivatized Hyp. BSA was used as negative control showing no peaks at m/z 412 (data not shown). At RTs 68.2 and 68.5 elute the isobaric species isoleucine and leucine, respectively

HUMAN COLLAGEN TYPE II



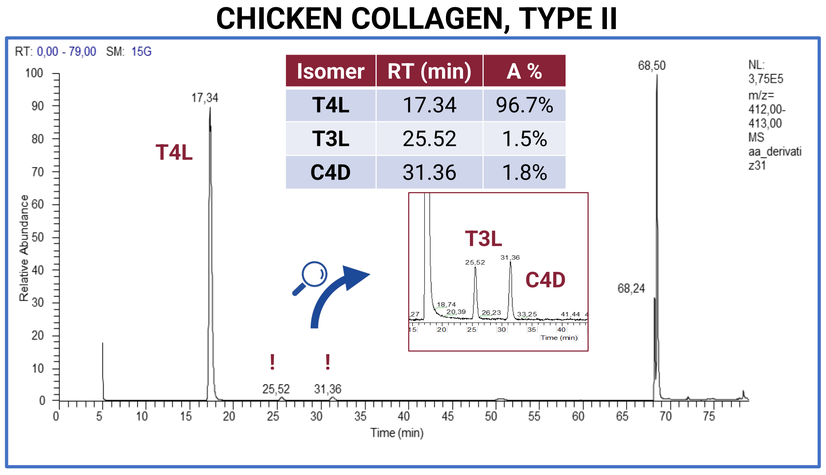
Isomer	RT (min)	A %
T4L	18.25	96.4%
T3L	26.86	1.5%
C4D	32.98	2.1%

RECOMBINANT HUMAN PROCOLLAGEN, TYPE II



Isomer	RT (min)	A %
T4L	17.14	85.9%
C4D	31.03	14.1%

CHICKEN COLLAGEN, TYPE II



Isomer	RT (min)	A %
T4L	17.34	96.7%
T3L	25.52	1.5%
C4D	31.36	1.8%

Data from chicken collagen and human collagen are according to the literature with T4L over 90% and less than 5% of its epimer and T3L, the second most abundant isomer in collagen. For recombinant procollagen however, the content of C4D is higher, probably due to the different folding status as compared as fibrillar collagen and the more accessibility of hydroxyprolines. Despite this, the high content of T4L in recombinant procollagen confirms the successful hydroxylation by P4H and highlights its specificity for 4-hydroxyproline, as evidenced by the absence of T3L