

A Rapid Quantification of Hydroxyproline in Leather using High-Performance Liquid Chromatography - Fluorescence Detection (HPLC-FLD) Method

by

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Abstract

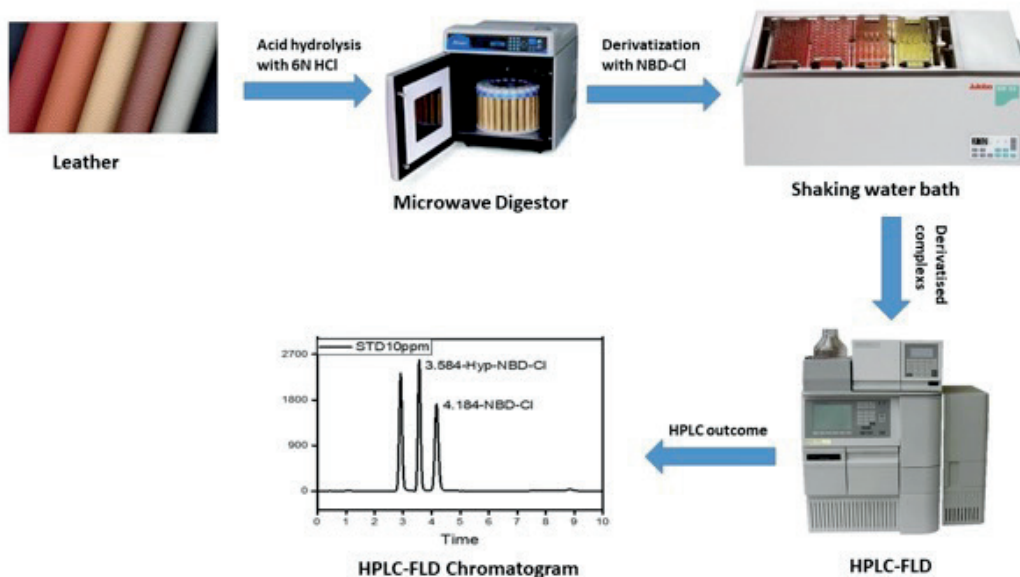
In the area of leather development research, determining collagen content through measurement of 4-Hydroxyproline (Hyp) has been major challenging task due to the interference of various leather-processing chemicals. To overcome this problem, for the first time we have used a High-Performance Liquid Chromatography coupled with Fluorescence Detection (HPLC-FLD) method for accurate determination of Hyp in leather samples through chemical derivatization of Hyp with 7-chloro 4 nitro benzofuran (NBD-Cl) reagent. The HPLC-FLD analysis was performed on a PICO TAG column with an isocratic mobile phase (80 % of 0.1M, pH 7.2 sodium acetate and 20% of acetonitrile, v/v) at a flow rate of 0.7mL/min. The detection was carried out at an excitation wavelength of 465 nm and its emission at 535nm. The retention time of Hyp was found to be ~3.5 minutes and the total run time was about 10 minutes. The method validation indicated that this analytical method is precise (3-12%RSD), accurate (90-100%), the limit of detection 0.01µg/ml, the limit of quantification - 0.03 µg/ml and linear (R^2 -0.9995) over the concentration range of 0.1 -2.0 µg/ml. The obtained result indicated that the assay linear range was acceptable for repeated analysis and

suitable for the complete range of hydroxyproline levels present in leather samples. Compared to the traditional method (IUC 17:1980), this analytical method demonstrates higher simplicity, specificity, reproducibility, and it could be useful for certifying leather products as well as inspecting international trade in leather and hides.

Introduction

Leather is made from animal skin and chemical tanning agents such as synthetic or natural chemicals.^{1,2} By cross-linking collagen molecules, a main structural protein found in the skin, these chemical tanning techniques improve the mechanical strength and stability of leather. Hydroxylated amino acids like 4-Hydroxyproline (Hyp) of about 12-14% are seen in long fibrous proteins namely collagen.^{3,4} For synthesis and maintenance of collagen's triple-helical structure, amino acids are vital. Several colorimetric methods for estimating collagen content of various samples by measuring Hyp have been developed over the years.⁵⁻⁷ The leather matrix includes a high concentration of salt due to its complicated nature. These salts, which are like chemical tanning agents, amino acids, fatty acids, and binders may generate considerable interferences and compromise

Graphical Abstract



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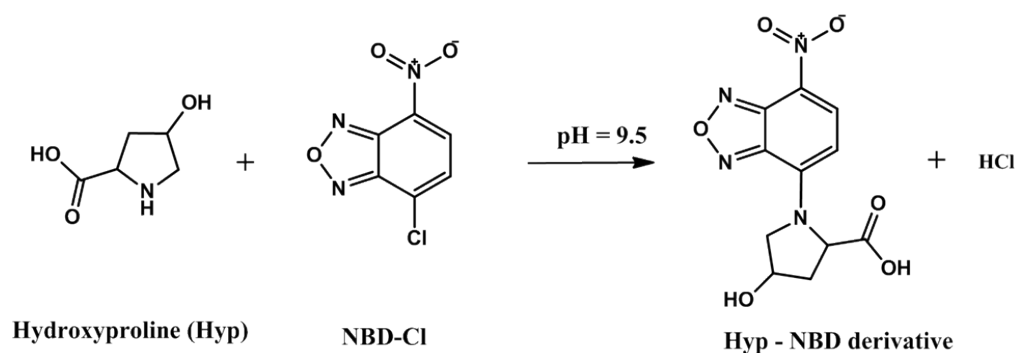


Figure 1. The general reaction between Hydroxyproline and NBD-Cl reagent.

the precise determination of Hyp. These calorimetric procedures are only applicable to pure collagen materials and not to leather samples. As a result, developing a precise method for quantifying Hyp in leather samples is crucial for the development of leather products.

Recently, numerous chromatography techniques have been used to measure Hyp from collagen content, which include high-performance liquid chromatography (HPLC), gas chromatography (GC), and micellar electrokinetic chromatography (MEKC).⁸⁻¹¹ The HPLC method is a simple, accurate, and cost-effective method for detecting Hyp in collagen-based products.⁹ The low sensitivity and selectivity, as well as the lengthier time required for sample preparation, limit the usefulness of HPLC method. Different pre-column derivatizing chemicals have been used in the HPLC process to improve sensitivity and selectivity.¹²⁻¹⁴ The following pre-column derivatizing agents are used such as phenylthiocarbamide (PTC), phenylisothiocyanate (PITC), 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl), O-phthalaldehyde (OPA), and 4-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS).^{8,12,15-19} These derivatized materials were analyzed via HPLC using various detectors like UV-visible absorption, fluorescence, electrochemical detection, and mass spectrometry.²⁰⁻²⁴ The most widely used derivatizing agent for quantifying Hyp is NBD-Cl. This is due to various biological samples that show excellent sensitivity, selectivity, and reactivity towards primary and secondary amines.^{18, 25} Furthermore, the NBD-Cl has a significant fluorescence property, which increases the capacity to detect the analyte even at low concentrations.⁹ However, because the fluorescent derivative based on NBD-Cl-Hyp is particularly light sensitive, it requires a longer chromatographic separation time of roughly 50 minutes.²⁶⁻²⁸ As a result, NBD-Cl is currently being investigated as a derivatizing reagent in an HPLC approach with a short chromatographic separation time.

To date the traditional colorimetric method (IUC17:1980) was the only method available to estimate the Hyp in leather.²⁹ In this method, Hyp was derivatized with p-Dimethylaminobenzaldehyde (DMAB) and oxidized to pyrrole. These traditional methods provide better sensitivity and selectivity for determination of Hyp in pure hide and untanned leather material. However, this method is not

suitable for tanned leather material due to requiring longer time for hydrolysis and involving a complicated oxidation reaction. This observation can be ascribed to the interference of numerous chemicals, which were used during the leather tanning process.

In this study hydrolysis time of leather into hydroxylates was reduced to 120 minutes and the proposed pre-column derivatization method for hydroxyproline quantification in leather, using NBD-Cl as a fluorescent labelling reagent for HPLC with fluorescence detection (FLD) was established. In accordance with CITAC Eura Chem requirement this method was validated.³⁰ The derivatization of hydroxyproline with NBD-Cl is depicted schematically in (Figure 1).

Experimental

Materials and Methods

Chemicals

Hydroxyproline (purity > 98%) and 7-chloro-4-nitrobenzol-2-oxa-1,3-diazole or NBD-Cl were purchased from Sigma-Aldrich Corporation, St. Louis MO, USA. NBD-Cl reagent (3000 µg/ml) was prepared in Methanol. Acetonitrile, Methanol, Dichloromethane and Tetrahydrofuran were of HPLC grade and procured from Merck Corporation Germany. Leathers of cow, buffalo, sheep and goat origin were collected from the pilot tannery of the institute. Hide powder was prepared as per standard procedure and characterized to meet international standards. All other chemicals used were of analytical grade without further purification. Water used for mobile phase and application was produced in the laboratory using Type I water purifier model Flex 3 of ELGA lab water UK.

Chromatographic conditions

A Waters e2695 HPLC-FLD system (Waters Instruments Corporation, Milford, USA) equipped with an alliance pump (2695 model), an autosampler and a multi λ fluorescence detector (2475 model) was used to analyze the hydroxyproline. The separation was performed on a PICO TAG column (3.9 x 300 mm 60A, 4µm) with an isocratic mobile phase comprising 80% of 0.1M sodium acetate and 20% of acetonitrile. The flow rate was fixed at 0.7 mL/min and 10µL of the sample was injected through Waters autosampler at

the temperature of 25°C. The fluorescence intensities of eluate were observed with a Waters fluorescence spectrophotometer (Model 2475) using an excitation wavelength of 465 nm and an emission wavelength in the range of 470 -800 nm respectively. Empower software, Version 2.0 (Waters Instruments Corporation, Milford, USA) was used to record and process the observed result. The recovery of hydroxyproline from the sample was calculated from the corresponding chromatogram peak area.

Preparation of Standard Hydroxyproline Solution

A stock solution of hydroxyproline (7.62 mM) was prepared by dissolving 100 mg of Hyp using acetonitrile (ACN) and water (H₂O) (1:1) mixture in 100 ml of standard flask. Standard solutions (5, 10, 25, and 50 µg/ml) were prepared by serial dilution of stock solution with ACN: H₂O mixture and used for the preparation of the calibration curve for the sample analysis.

Preparation of Sample and derivatization

Leather (2 g, width 4mm × height 4mm) was taken in a 250 mL amber bottle and 50 mL of dichloromethane (DCM) and tetrahydrofuran (THF) solvents mixture (1:1 ratio) was added for the leather degreasing process. Subsequently, these solutions were ultrasonicated for 30 minutes at room temperature to remove the fat and top finishing chemicals present in leather. This degreased leather was taken out from an amber bottle and dried in a hot air oven at 50°C for 6 h to remove the adsorbed solvents. The dried leather sample was placed in a 100 mL amber bottle and 15 mL of Type-1 water was added and kept in a shaker for 30 minutes at 60 rpm to remove the soluble metals in the degreased leather. Following this, the leather was dried in a hot air oven at 120°C for 10 minutes and kept in a desiccator till further work. From the desiccator, 50 mg of degreased leather sample was taken in a glass vessel employed with microwave digester CEM Corporation model MARS (USA) and 10 ml of hydrochloric acid solution (6 N) was added for the hydrolysis process. The microwave-based hydrolysis reaction was performed with 100 psi at 120°C for 120 minutes. After this, the hydrolysate, which was obtained, was made up to 50mL by using Type-1 water. Ten mL of diluted hydrolysate was pipetted into a China dish and dried completely over the water bath at 50°C for 60 minutes. The obtained yellowish residue was re-dissolved and made up to 10mL by using 0.4M borate buffer and stored in the refrigerator at 4°C until use.

For the derivatization procedure, an aliquot of the hydrolyzed sample (100 µL) or standard stock solution (50-500 µL) was taken in a 10mL standard flask and 0.5mL of NBD-Cl reagent was added (3000 µg/ml in methanol) along with 1 mL of 0.4N borate buffer (pH = 9.5) and was made up to completion by adding methanol. The resulting mixture was transferred to a 25mL polypropylene centrifuge tube and placed in a shaking water bath at 60°C for 5 minutes. To this, 100 µL of 1N HCl solution was slowly added and cooled to 0°C for 30min. The obtained final solution was filtered through Whatman

filter paper number 42 and further filtered with 0.2 µm Millipore hydrophilic syringe filter paper, injected into HPLC-FLD. The obtained results from the leather samples were compared against standard collagen and hide powder.

Method Validation

The proposed chromatographic method was validated according to Eurachem CITAC guidelines determining linearity, limits of detection (LOD), limit of quantification (LOQ), Precision, Accuracy and Robustness.

Linearity and concentration range

The linearity of the chromatographic method was tested for Hyp-NBD derivatives. The linearity was studied within the concentration range of 0.1-2.0 µg/mL for Hyp. The calibration curves were constructed by plotting fluorescent peak area against concentrations of Hyp. The obtained data were statistically treated using the linear regression analysis and the analytical parameters were calculated.

Limit of detection and quantification

The limits of detection (LOD) and quantification (LOQ) of Hyp were determined at respective signal-to-noise (S/N) ratios of 3 and 5 by repeated analysis (n=10) of the same concentration of Hyp solution. LOD and LOQ were calculated as $3 \times sA/B$ and $10 \times sA/B$ respectively, where sA is the standard deviation of response and B is the slope of the linear equation used for calibration.

Accuracy

Accuracy of the chromatographic method was determined by spike recovery studies at three different concentration levels (30%, 100% and 200% is concentration level reported in percentage) of Hyp within the LOQ range. Three samples from each concentration level were analyzed and the average result is presented as a percentage of relative spike recovery.

Precision

Precision (Inter-day) of the chromatographic method was assessed by six repeated analyses of three different concentration levels (30%, 100% and 200%) of Hyp within the LOQ range. The obtained precision result is represented as a percentage of relative standard deviation.

Robustness

The robustness of the proposed method was assessed by evaluating the influence of six different analysts and obtained results are expressed in relative standard deviation.

Estimation of Fibrous protein in the tanned leather sample

The fibrous protein (collagen) content in leather samples were estimated from the hydroxyproline content of leather hydrolysate. The mass ratio of collagen to hydroxyproline in leather sample was calculated using a factor ranging from 7.14-7.69. The percentage of

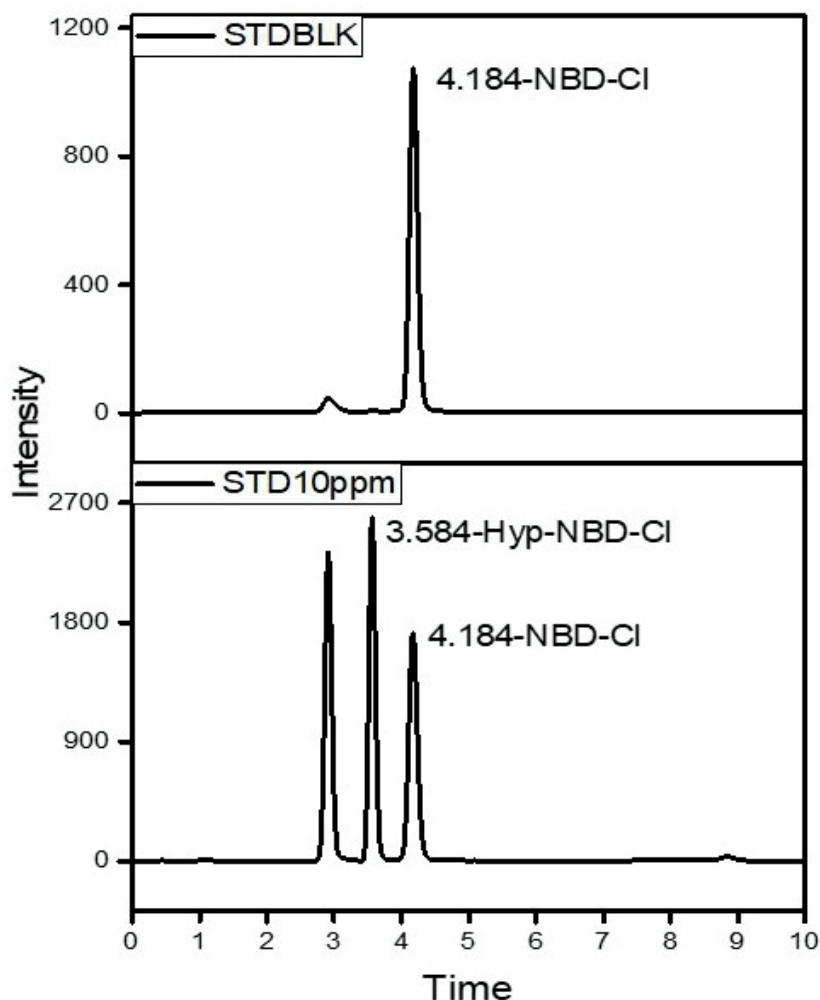


Figure 2. HPLC chromatogram of Blank and Standard Hydroxyproline.

fibrous protein in leather sample can be calculated through using equation 1.^{31,32}

$$\frac{\text{Micrograms hydroxyproline in 1 mL hydrolysate}}{\text{Micrograms leather represent in 1 mL hydrolysate}} \times 7.46 \times 100 \quad (1)$$

Results and Discussion

Method development and optimization

The prepared derivatized sample (standard Hyp-NBD) was injected in the HPLC-FLD system and isocratic mobile phase separation was detected in fluorescence and chromatogram system (Figure 2). In fluorescence spectrum, the appearance of broad emission at 535nm indicated that NBD-Cl was successfully derivatized with Hyp. This observation was further confirmed by the HPLC chromatogram. The derivatized hydroxyproline exhibited several elution peaks with higher symmetry and resolution. The elution peaks appeared at a retention time of 2.913, 3.584 and 4.194 minutes corresponding to blank, Hyp-NBD-Cl and un-reacted NBD-Cl respectively. The absence of co-elution along with Hyp peak suggested that this developed method has higher specificity in the determination of hydroxyproline. To find the suitable derivative reaction and separation

condition for the HPLC-FLD method, various experimental parameters including column type, mobile phase composition, pH of the buffer, the concentration of NBD-Cl, reaction temperature and time of the derivative reaction were investigated.

Derivatization of standard hydroxyproline with NBD-Cl was performed in acidic and basic pH conditions. The results of the experiment indicated that Hyp-NBD based fluorescent emission peak appeared only at pH above 9.5, whereas no emission peak was observed in acidic (pH 2.2) conditions. This result clearly suggested that derivatization specifically occurred in Hyp at basic pH conditions through SN2 nucleophilic reaction between the Hyp and NBD-Cl reagent.^{28,30}

Three column materials, viz., Gemini (18) (5 μ), Chromolith RP-18e and PICO TAG were used to separate the Hyp-NBD derivatives. The obtained chromatogram indicated that the PICO TAG column showed better separation compared to the other two columns.

The role of the mobile phase was investigated by varying the composition. Sodium acetate and acetonitrile were used as mobile phases at 60:40, 70:30 and 80:20 v/v. A better separation

Table I
Optimization of chromatographic method through changing the various parameters.

Columns used	Mobile Phase	Flowrate (mL/min)	Observation	Result
Gemini (18) (5 μ) (110A $^\circ$) 150 \times 4.60mm	(Sodium Acetate: Acetonitrile) 60:40 70:30 80:20	0.7 - 1.0	Not Proper Separation	Method Rejected
Chromolith $^\circ$ RP-18e 2 μ M, 130 Å	(Sodium Acetate: Acetonitrile) 60:40 70:30 80:20	0.7 - 1.0	Not Proper Separation and variable RT	Method Rejected
PICO TAG, 3.9 \times 300mm 60A, 4 μ m	(Sodium Acetate: Acetonitrile) 60:40 70:30 80:20	0.7	Proper Separation	Method Accepted

of hydroxyproline was observed at sodium acetate to acetonitrile ratio of 80:20 v/v.

The time and temperature of microwave hydrolysis play an important role in the recovery of Hyp. Initially, the microwave hydrolysis was carried out for 30 minutes at 110 $^\circ$ C and the Hyp recovery was around 3-5% in hide powder. When microwave hydrolysis was extended for 60 minutes at 115 $^\circ$ C, Hyp recovery increased to 8-10%. Further extension of microwave hydrolysis to 120 min at 120 $^\circ$ C leads to 12-13% of Hyp recovery.

The influence of NBD-Cl volume (5 μ L, 10 μ L and 100 μ L i.e, 7.62 μ M, 38.1 μ M and 76.2 μ M) in the recovery of Hyp was analyzed. Hyp recovery was around 5, 25 and 95% with 5, 10 and 100 μ L of NBD-Cl solution respectively. This result indicated that the reaction of NBD-Cl with Hyp is mostly completed when the concentration of NBD-Cl is 150 times that of the Hydroxyproline.

The optimum conditions were defined as: pH of borate buffer 9.5, NBD-Cl of volume -100 μ L, mobile phase containing 80:20 v/v sodium acetate: acetonitrile, flowrate 0.7mL/min, reaction temperature and time of 120 $^\circ$ C and 120 minutes respectively. The characteristic retention time was around \sim 3.5 minutes for the Hyp-NBD derivative. The result of the method optimization is summarized in Table I.

Method validation

Linearity

The obtained calibration curve is presented in (Figure 3). The results from the linearity study indicated a linear relationship in the concentration range of 0.1-2.0 μ g/ml for the Hyp-NBD derivative. From the regression analysis, a linear equation $y = 3.00 \times 1007(x) - 1.66 \times 1006$, where y is the fluorescent peak area and x

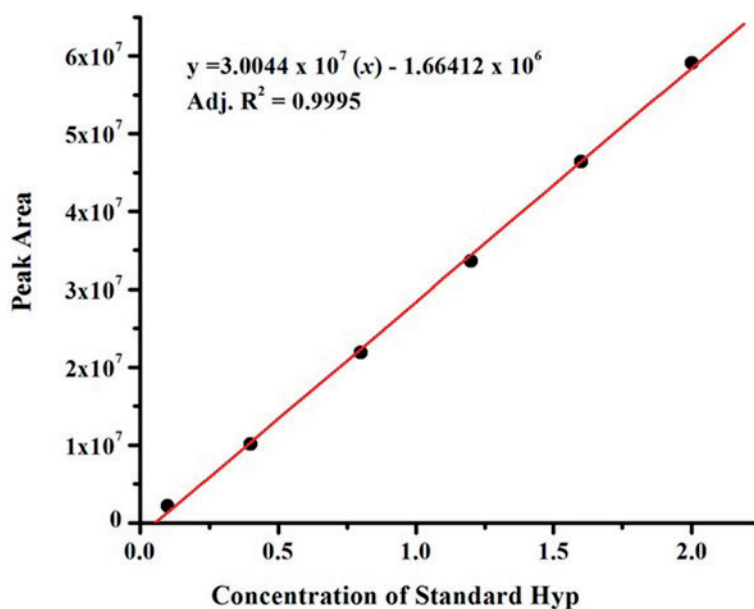


Figure 3. Linearity curve of standard Hyp-NBD-Cl.

Table II
Summary of parameters in Method Validation

Parameter		Hydroxyproline	Acceptance Criterion	
LOD	µg/ml	0.01	-	
LOQ	µg/ml	0.03	-	
Linearity	R ²	0.9995	R ² value is ≥0.99	
Accuracy	30% LOQ	Average Recovery (%)	Values found to be within the range of 70-120%	
	100% LOQ			96.3
	200% LOQ			99.9
Method Precision	30% LOQ	RSD	RSD value was found to be ≤20%	
	100% LOQ			113.6
	200% LOQ			9.4
Robustness	RSD	7.0	RSD value was found to be ≤20%	

is the concentration of Hyp has been established. The correlation coefficient (R²) was found to be 0.9998, indicating the excellent linearity of the proposed method.

LOD and LOQ

The Limit of Detection (LOD) and Limit of Quantification (LOQ) were assessed based on the respective signal to noise ratios of the standard Hyp (Table III). The LOD and LOQ were found to be

Table III
Limits of detection (LOD) and quantitation (LOQ) of proposed method

Number of Injections	Area
1	570745
2	401692
3	432223
4	659130
5	519687
6	400695
7	691522
8	573333
9	506866
10	512869
Mean	526876.2
St.dev(sA)	99934.6
B(Slope)	30044194
LOD=3X(sA)/B	0.01
LOQ=10X(sA)/B	0.03

0.01 and 0.03 µg/mL respectively. The observed LOD value was much lower than the previously reported method (LOD <1 µg/ml and LOQ 5.5 µg/ml).³³ The LOQ of Hyp-NBD-Cl obtained by the proposed system was compared with the previous reports and the results clearly indicated a higher sensitivity for Hyp analysis.

Accuracy

The accuracy was determined based on the recovery of Hyp and the results were expressed as the relative spike recovery for three different percentage solutions (Table IV). The obtained results showed that spike recovery ranges from 99.89% to 113.61%. The obtained results showed good agreement between the measured and actual value (70-120%) indicating the high accuracy of the proposed method.

Precision

Inter-day precision was examined at three different concentration levels and the obtained results are summarized in Table V. The measured relative standard deviations were below 20% indicating an excellent precision of the proposed method.

Robustness

The results of robustness analysis indicated that a change of analyst did not significantly affect the percentage of RSD values. The robustness results are summarized in Table VI. The obtained percentage of RSD values were within the acceptable limits (RSD < 20 %) which clearly suggested that the proposed chromatographic method is highly reliable under the optimized condition. Summarization of the parameter in method validation is given in Table II.

Table IV
Precision of the proposed method

Analyte	% Of LOQ (Number of Injections)	Injection values (Area)	Mean	Standard deviation	RSD
Hyp	30% of LOQ				
	1	137739	143875	13515	9.4
	2	148667			
	3	132508			
	4	161223			
	5	155906			
	6	127209			
	100% of LOQ				
	1	570745	529029	37251	7.0
	2	551692			
	3	492223			
	4	559130			
	5	519687			
	6	480695			
	200% of LOQ				
	1	779530	724377	58830	8.1
	2	688695			
	3	793335			
4	662538				
5	756118				
6	666043				

Table V
Accuracy of the proposed method

Analyte	% Of LOQ	Spike Conc. ($\mu\text{g/ml}$)	Sample Conc. ($\mu\text{g/ml}$)	Actual Conc. ($\mu\text{g/ml}$)	Recovery	Average (%)
Hyp	30% of LOQ	0.01	0	0.008	80.0	96.3
		0.01	0	0.0114	114.0	
		0.01	0	0.0095	95.0	
	100% of LOQ	0.03	0	0.0295	98.3	99.9
		0.03	0	0.0323	107.7	
		0.03	0	0.0281	93.7	
	200% of LOQ	0.06	0	0.0853	108.8	113.6
		0.06	0	0.0707	117.8	
		0.06	0	0.0885	114.2	

Table VI
Robustness of the proposed method

Analyte	100 % Of LOQ (Number of Injections)	Injection values (Area)	Mean	Standard deviation (sA)	RSD
Hyp	1	570745	529029	37251	7.0
	2	551692			
	3	492223			
	4	559130			
	5	519687			
	6	480695			

Tanned leather sample

The optimized chromatographic condition was applied to the various animal origin leather samples to determine the hydroxyproline content in the leather. The obtained results (Figure 4) indicated that the retention time of Hyp was around 3.5 minutes for all leather samples. This observation clearly suggested that the retention time of hydroxyproline in leather samples was not significantly changed in the presence of various chemical interferences when compared to pure collagen samples. The quantity of Hyp and collagen content in leather samples is presented in Table VII. In addition, the quantified Hyp content in the leather sample matched with pure collagen and hide powder (Hyp, 12-14%).³⁴ The obtained result indicated that there is an insignificant difference in the Hyp content based on the animal origin. Therefore, future work will focus on the differentiation of leather samples through the measurement of hydroxyproline content in advanced spectral techniques.

Conclusion

In this study, we have demonstrated the applicability of the HPLC-FLD method to accurately determine the hydroxyproline in leather samples. The developed method was validated according to Eurachem CITAC guidelines. The main feature of the developed method is the short retention time of around 3.5 minutes. This method is simple, sensitive, linear, precise, accurate, reproducible and readily applicable to leather from different animal origins. This method is versatile in quantification of animal protein in leather

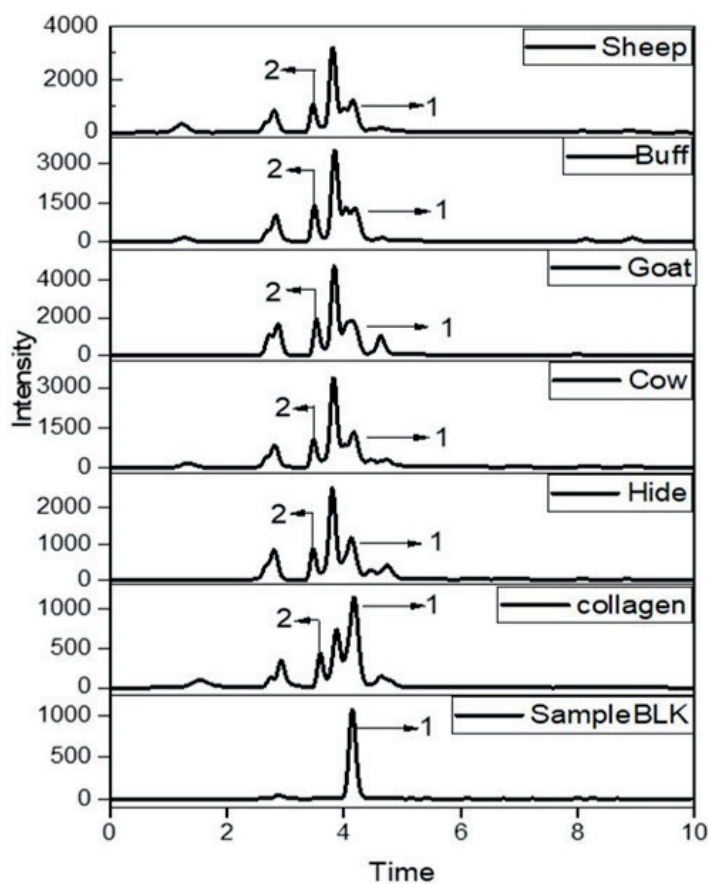


Figure 4. HPLC chromatogram of collagen, Hide and different origin leather samples, Where 1 denotes the retention time of NBD-Cl and 2 denotes the retention time of Hyp-NBD-Cl complex.

Table VII
Total collagen content calculated using the equation

S.No.	Specimen	Hydroxyproline Content	Total collagen Content
1.	Collagen-Type-I	0.136	97.92
2.	Hide Powder	0.132	95.04
3.	Buff leather	0.092	66.24
4.	Cow leather	0.097	69.84
5.	Sheep leather	0.098	70.56
6.	Goat leather	0.097	69.84

using hydroxyproline estimation. This method can be used to distinguish leather from vegan based leather-like materials such as those from cactus, pineapple etc., and leather like material based on the polymeric material. This study can establish the genuineness of the leather product.

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Declaration of interest statement

Priya Narayanan, Suresh Sethurajan, Mohan Vedhanayagam, Kalarical Janardhanan Sreeram all the authors are full time employees of CSIR-Central Leather Research Institute. None of the authors have conflicts to declare.

Author contribution

Priya Narayanan: Conceptualization, Methodology, Validation, and Writing - Original Draft, Suresh Sethurajan: Data Curation, Software, Vedhanayagam: Writing - Review & Editing Kalarical Janardhanan Sreeram: Visualization, Investigation, Supervision.

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