

# DETERMINATION OF HYDROXYPROLINE USING ION-EXCHANGE LIQUID CHROMATOGRAPHY WITH VIS DETECTOR AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTOR

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*Abstract:* The first aim of the experiment was an optimization of the method for determination of hydroxyproline in a pig skin using high-performance liquid chromatography with fluorescence detector (HPLC-FLD) and ion-exchange liquid chromatography with VIS detector (IEC-VIS). On the basis of the experiments performed, it was found that HPLC-FLD method is three times more sensitive than IEC-VIS method. For IEC analysis of hydroxyproline, the limit of detection (LOD) was 4.10 µg/ml whereas the limit of quantification (LOQ) was 13.50 µg/ml. For HPLC analysis of hydroxyproline, the limit of detection (LOD) was 1 ng/ml whereas the limit of quantification (LOQ) was 3 ng/ml. The second aim was the testing of influence of different volume of 6M HCl on extraction of sample (50 mg) for analysis using HPLC-FLD. Here it was found that the best volume was 250 µl 6M HCl.

*Key Words:* hydroxyproline, HPLC-FLD, IEC-VIS, microwave hydrolysis, pig skin

## INTRODUCTION

Hydroxyproline (C<sub>5</sub>H<sub>9</sub>O<sub>3</sub>N) is a non-proteinogenic amino acid produced by hydroxylation of the amino acid proline via prolyl hydroxylase enzyme (Gorres and Raines 2010). Hydroxyproline and proline are a main part of collagen of animals (Zhang and Duan 2017) and humans (Sugioka et al. 2017). Hydroxyproline organize the triple-helical structure of collagen and played key role in collagen stability (Shoulders and Raines 2009). Content of hydroxyproline in biological fluids is used as a parameter of collagen catabolism (Lv et al. 2017), especially bone resorption or tissue degradation (Kimura et al. 2017). Different changes in hydroxyproline metabolism play major roles in the pathophysiology and pathogenesis of various diseases (Srivastava et al. 2016). The elevated level of hydroxyproline was observed in several disorders, i.e. graft versus host disease, keloids, vitiligo, cases of depression and stress (Srivastava et al. 2016, Dong et al. 2017). Decreased level of hydroxyproline is a marker of poor wound-healing (Srivastava et al. 2016). Degradation of collagen may accelerate the increased amount of reactive oxygen species (ROS) (Shi et al. 2016). Hydroxyproline is a potential oxidative biomarker for diagnosis of fibrosis in hepatitis C (Attallah et al. 2007), adiposity and cardiometabolic health (Jennings et al. 2016).

Several methods are usually used for determination of hydroxyproline from different types of matrices, including high-performance liquid chromatography with fluorescence detection (Ren et al.

2017), high-performance liquid chromatography with tandem mass spectrometric detection (LC/MS/MS) (Shiota et al. 2017), hydrophilic interaction chromatography with tandem mass spectrometry and ion-exchange liquid chromatography with post-column derivatization (Rigas 2012, Zhu et al. 2014).

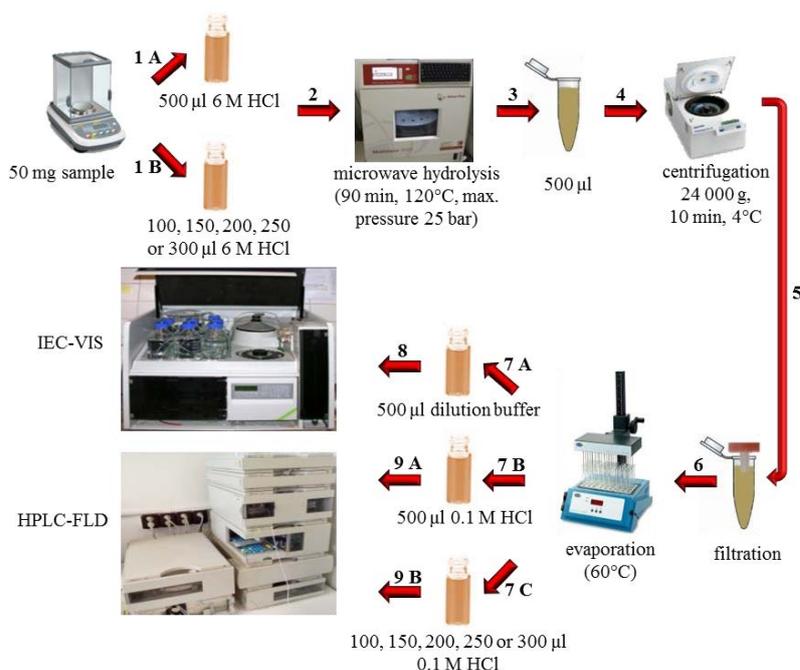
We have focused this work on optimization of two chromatographic methods, namely, ion-exchange liquid chromatography with VIS detector and high-performance liquid chromatography with fluorescence detector.

## MATERIAL AND METHODS

A pig skin was used in this experiment. The preparation of samples for high-performance liquid chromatography with fluorescence detector (HPLC-FLD) and ion-exchange liquid chromatography with VIS detector (IEC-VIS) were a bit different. For the first aim of this experiment, the sample preparation included the following process numbers (Figure 1): 1 A, 2, 3, 4, 5, 6, 7 A, 7 B, 8 and 9 A. As an alternative sample preparation procedure, we chose different volumes of 6M HCl for sampling 50 mg of a sample. This sample preparation method was used for HPLC-FLD analysis. For the second aim of this experiment, the sample preparation included the following process numbers (Figure 1): 1 B, 2, 3, 4, 5, 6, 7 C and 9 B.

The dilution buffer consisted of thiodiglycol (5 ml/l), sodium azide (0.1 g/l), citric acid (14 g/l) and sodium chloride (11.5 g/l).

*Figure 1* Scheme of the pig skin sample preparation for IEC-VIS and for HPLC-FLD analysis and scheme of optimization of the pig skin samples preparation for HPLC-FLD analysis



For the primary determination of hydroxyproline, an ion-exchange liquid chromatograph AAA-400 (Ingos, Czech Republic) with post column derivatization by ninhydrin and an absorbance detector in visible light range (VIS) were used. A glass column with an inner diameter of 3.7 mm and a length of 350 mm was filled manually with strong cation exchanger (LG ANB, Ingos, Czech Republic) with ~12 µm particles and 8% porosity. The column was equilibrated at 60 °C. A double-channel VIS detector with an inner cell of 5 µl was set to two wavelengths: 440 and 570 nm. A prepared solution of ninhydrin was stored under nitrogen atmosphere in the dark at 4 °C. The elution of amino acids was carried out by buffer containing 10.0 g of citric acid, 5.6 g of sodium citrate, and 8.4 g of sodium chloride per one liter of solution (pH 2.7). The flow rate was 0.25 ml/min. The reactor temperature was set to 120 °C. All chemicals were purchased from Ingos (Ingos, Czech Republic).

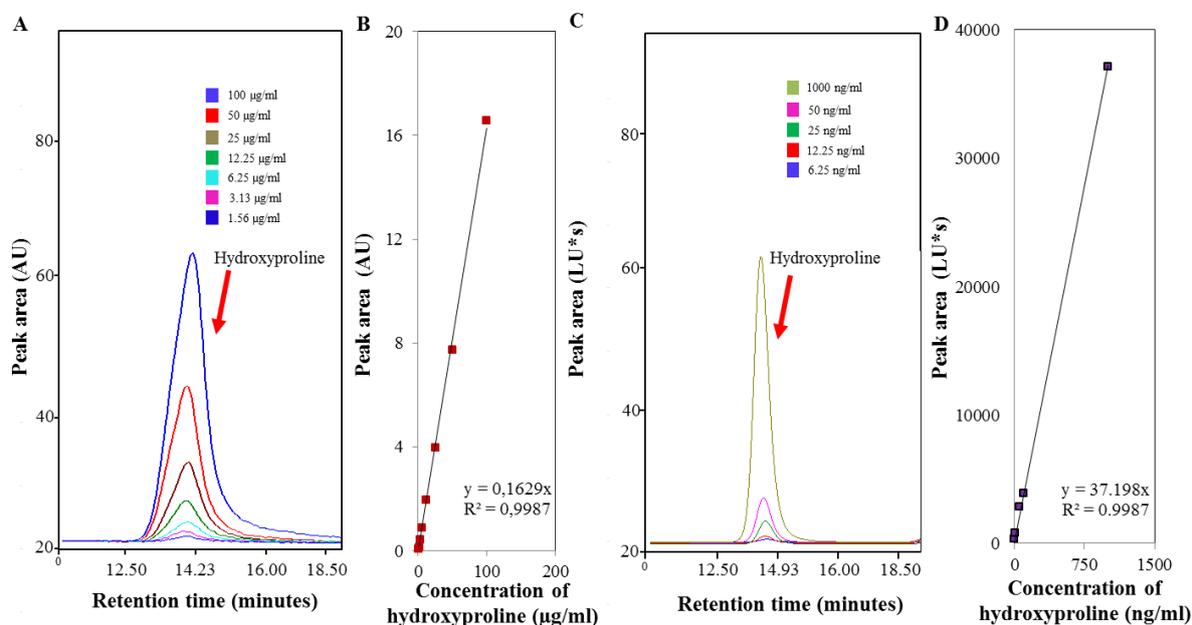
For alternative determination of hydroxyproline, a high-performance liquid chromatograph HP 1100 Series with FLD detector (HP, Germany) was used. The HPLC chromatographic system was controlled with ChemStation software (rev. A 07.01). The column effluent was monitored with a diode-array detector at 338 nm (10 nm bandwidth) and a fluorescence detector at 340<sub>ex</sub>/450<sub>em</sub> nm using the OPA reagent for precolumn derivatization. For the separation of amino acids, the column Zorbax Eclipse AAA (Agilent Technologies, USA) with dimensions 150 x 4.6 mm and a particle size of 3.5 µm was used. The column was equilibrated at 40 °C. A mobile phase A consisted of 40 mM Na<sub>2</sub>HPO<sub>4</sub> at pH 7.8 (5.5 g of NaH<sub>2</sub>PO<sub>4</sub> monohydrate + 1 l of H<sub>2</sub>O, adjusted to pH 7.8 with 10M NaOH solution) and a mobile phase B was acetonitrile/methanol/water (45:45:10 v/v). The flow rate of mobile phase was 2 ml/min. The compounds were eluted with a linear upward gradient: 0.0 min (0% B) 0.8 min (57% B) 10.0 min (100% B) 12.5 min (0% B) to 14.0 min. All chemicals were purchased from Sigma-Aldrich (St. Louis, USA).

## RESULTS AND DISCUSSION

A determination of the presence and concentration of hydroxyproline was held by a high-performance liquid chromatography with fluorescence detection and an ion-exchange liquid chromatography with VIS detector. The content of hydroxyproline in a pig skin was expressed as mean ± standard deviation from three replicates.

Two calibration curves were prepared in the ranges 1.56 – 100.00 µg/ml for IEC-VIS and 6.25 – 1000.00 ng/ml for HPLC-FLD. The calibration curve for IEC-VIS showed a good linearity with correlation coefficient  $R^2 = 0.9987$  and R.S.D. = 2.1% (Figure 2A, B). The calibration curve for HPLC-FLD showed a good linearity with correlation coefficient  $R^2 = 0.9987$  and R.S.D. = 1.75% (Figure 2C, D).

Figure 2 (A) Chromatograms from calibration dependence of hydroxyproline determined by IEC-VIS, (B) calibration curve measured within the range from 1.56 to 100.00 µg/ml under the experimental conditions, (C) chromatograms from calibration dependence of hydroxyproline determined by HPLC-FLD, (D) calibration curve measured within the range from 6.25 to 1000.00 ng/ml under the experimental conditions



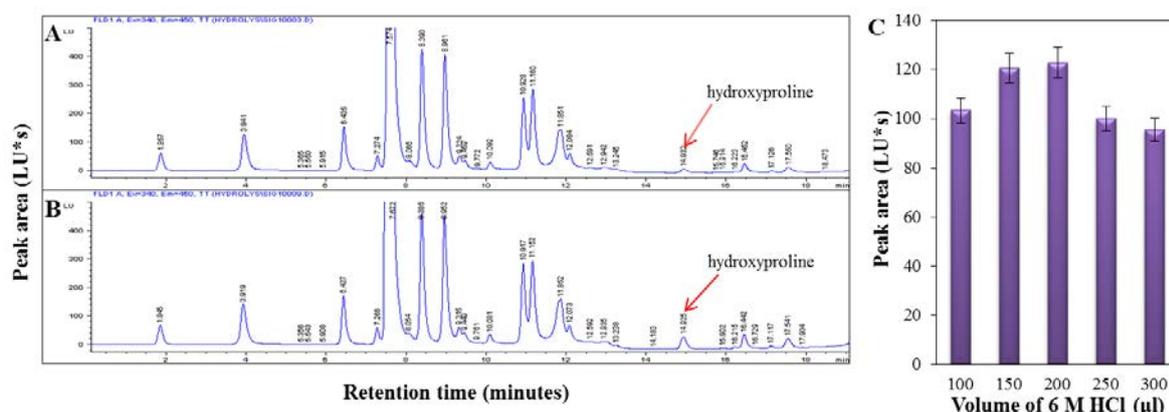
After comparing the results of HPLC-FLD and IEC-VIS methods, it was clear that HPLC-FLD provided higher assay sensitivity than IEC-VIS. A higher sensitivity of HPLC-FLD is demonstrated by a calibration curve where the calibration range is in an order of ng/ml, whereas the calibration range for IEC-VIS method is in an order of µg/ml. Another advantage of HPLC-FLD is that FLD is a selective detector and therefore HPLC-FLD could be used as a confirmatory method. HPLC-VIS

results always need to be confirmed using any other method, for example LC-MS. This may be the subject of further experiments. Another advantage is the sample volume, which is 250  $\mu\text{l}$  for IEC-VIS and 5  $\mu\text{l}$  for HPLC-FLD, respectively. Therefore, only HPLC-FLD was used for further experiments. The initial preparation of the sample was replaced and different volumes of 6M HCl were optimized for a load of 50 mg of a sample.

The representation of influence of different volumes of 6M HCl for mineralization of a pig skin sample is shown in figure 3C. For each vial containing 50 mg of a pig skin, the following volumes of 6M HCl were added: 100, 150, 200, 250 and 300  $\mu\text{l}$ . The results shown in figure 3C indicate that the best yield of hydroxyproline was acquired using a volume of 200  $\mu\text{l}$  of 6M HCl compared to the other volumes of 6M HCl.

In figures 3A and 3B, the HPLC-FLD chromatogram of real samples of a pig skin is shown. The chromatogram in figure 3A shows the presence of hydroxyproline in a 50 mg real sample dissolved in 200  $\mu\text{l}$  of 6M HCl. Hydroxyproline concentration was 4 ng/ml. The chromatogram in figure 3B shows the presence of hydroxyproline in a 50 mg real sample dissolved in 200  $\mu\text{l}$  of 6M HCl spiked with 10 ng/ml of a standard hydroxyproline. Hydroxyproline concentration was 14 ng/ml.

*Figure 3 Chromatogram of real sample of a pig skin (50 mg), mineralized in 200  $\mu\text{l}$  of 6M HCl, (A) without spike and (B) spiked with standard of hydroxyproline (10 ng/ml). (C) Influence of added different volume of 6M HCl to the 50 mg of real samples of a pig skin on the concentration of hydroxyproline after microwave mineralization.*



## CONCLUSION

Based on the experiment, the comparison of two tested methods, HPLC-FLD and IEC-VIS, showed that the HPLC-FLD method is much more sensitive than the IEC-VIS method for the detection and quantitation of hydroxyproline. The HPLC-FLD sensitivity versus IEC-VIS sensitivity was three orders better ( $\mu\text{g}/\text{ml}$  versus  $\text{ng}/\text{ml}$ ). Another advantage is the used sample volume, which is 250  $\mu\text{l}$  for IEC-VIS and 5  $\mu\text{l}$  for HPLC-FLD. From the results of the optimization of the 6M HCl volume used in dilution of 50 mg of a real sample, it was found that the best for the highest yield of hydroxyproline from the real sample was a 200  $\mu\text{l}$  of 6M HCl. The hydroxyproline concentration 4 ng/ml in the real 50 mg sample was optimized with the use of 200- $\mu\text{l}$  6M HCl. It was proved, that HPLC-FLD is a suitable method for analysis of hydroxyproline in low concentrations and sample amounts and can be used as a detection method for analysis of real samples of a skin for further experiments.

## ACKNOWLEDGEMENT

This research was carried out under the project CEITEC 2020 (LQ1601) with financial support from the Ministry of Education, Youth and Sports of the Czech Republic under the National Sustainability Programme II and MENDELU IGA TP3/2017.

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