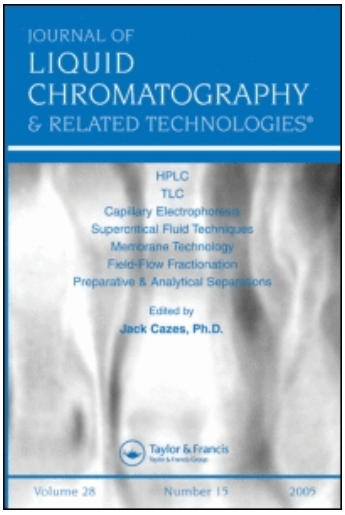

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Hydroxyproline Measurement by HPLC: Improved Method of Total Collagen Determination in Meat Samples

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ABSTRACT

A sensitive and selective assay of hydroxyproline is presented for the determination of collagen in meat and meat products. The amino acid is converted to a sensitive fluorescent derivative with 7-chloro-4-nitrobenzofurazan (NBD-Cl), followed by reversed-phased chromatography and fluorescence measurement (ex = 465 nm, fl = 535 nm). Hydroxyproline and proline can be measured by this system. The column and detector performance shows good retention, selectivity, and efficiency. Based on replicated analysis of controls over a range of 0–20 mg/mL, the

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method is accurate (94–100%) and precise (%CV of 0.3–13). The limit of quantification for hydroxyproline and proline was 0.0027 ng/mL. Fourteen meat samples with different levels of collagen normally found in meat and meat products were tested. The results showed that the linear range of assay was suitable for routine analysis, and applicable to the entire range of hydroxyproline levels normally found in meat and meat products (0.05–12.5%).

Key Words: Hydroxyproline; Collagen; Meat samples; Amino acids.

INTRODUCTION

There is a great need for a satisfactory method for the determination of hydroxyproline to facilitate the study of the composition of meat proteins. The unique, high hydroxyproline content of collagen suggests the desirability of an accurate method for the determination of this amino acid in small quantities as a means of estimating the amount of collagen or gelatin in a mixture of proteins.[1]

Analysis of amino acids with ninhydrine reaction gives an extremely small peak in comparison with other amino acids. This is due, in part, to the relatively low absorbance coefficient obtained with this reagent. Until recently, the most popular approach for the analysis of collagen in meat and meat products were the hydroxyproline colorimetric procedures of Stegemann or Newman and Logan.[1–9]

Measurement of collagen concentration in meat usually involves overnight hydrolysis of a homogenate in concentrated hydrochloric acid, neutralization of the acid, and finally, colorimetric assay of the amino acid hydroxyproline. There are, however, numerous problems associated with this approach, as evidenced by the various modifications.[10–13] Fluorescamine and *o*-phthalaldehyde result in high fluorescence intensity with α-amino acids, but none with secondary amino compounds. HPLC methods have been reported to determine hydroxyproline in different samples,[11,14,15] but only a few in meat and meat products.[16–18] The reagent, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole or NBD-Cl, provides much higher fluorescence intensities with amino acids than with α-amino acids.[19,20] The hydroxyproline determination could be made more sensitive, specific, and accurate, by using the high-performance liquid chromatography and derivatization with NBD-Cl. NBD-Cl reagent, coupled with HPLC, has been introduced to overcome problems of sensitivity and specificity and/or long time analysis in non-meat samples.[14,21–23]

The objective of the present study, was to combine the separating power of the reversed-phase chromatography with the high sensibility of the NBD-Cl fluorometric detection for the determination of proline and hydroxyproline in meat and meat products. The present report describes an assay method to provide a rapid, sensitive procedure applicable to a wide range of hydroxyproline levels in meat and meat products.

EXPERIMENTAL

Sample Preparation

Standard methods from the AOAC[24] were followed for the sample preparation. All samples were homogenized in a waring blender (VWR). A whole sample of 200 g was dried at 100°C for 16 hr (Sections 934.01, 950.46), defatted with petroleum ether for 6 hr (Sections 920.135, 900.002 A), and then grounded in a Cyclotec (Tecator 1092) mill with 80 mesh.

Hydrolysis

A sample of 3.0($\pm$0.4) mg was hydrolyzed with 2.0 mL of HCl 6 M (laboratory prepared or Pierce 24309) in a Vacuum Hydrolysis Tube (Pierce 29560) at 150 ($\pm$5)°C for 6 hr in a Vacuum Reaction System (Pierce 8870). Samples were evaporated under vacuum at 65 ($\pm$1)°C (Brinkmann Büchi RE 121) and suspended with 2.0 mL 0.2 N sodium citrate sample dilution buffer, pH 2.2 (Pierce Cat No. 27216).

Sample Derivatization

An aliquot of 250 μL of the hydrolyzed sample was diluted to 1.0 mL with 0.4 M borate buffer, pH 10.4 (Pierce 27035); 250 μL of the dilution was mixed with 250 μL of NBD-Cl (7-cloro-4-nitrobenzo-2-oxa-1,3-diazol, $C_6H_2ClN_3O_3$, Sigma C-5261) solution (2.0 mg/mL in methanol), and the mixture was heated for 5.0 min at 60 ($\pm$1)°C in a closed screw capped vial. The reaction was stopped by adding 50 μL of HCl 1 M (laboratory prepared) and cooled immediately at 0°C for 30 min, and 10 μL was injected onto the column. Standards and samples were injected every 15 min.

HPLC Instrumentation

The liquid chromatographic system (Varian, Palo Alto, CA) consisted of a Varian Model 9012 high-pressure solvent delivery pump, a Varian Fluorichrom II detector set at 330 and 418 nm cut-off filters, and a 7125 manual injector with 10 μL fixed loop (Rhedyne, Cotati, CA). The analysis was performed using a Microsorb short-One Varian/Rainin analytical commercial column (89-200-E3) 10 cm × 4.6 mm ID, C_{-18}, 3 μm particle size, 100 Å. The chromatograph system was connected to a Varian Star Chromatography Software Version 4.0, and peak areas were reported.

Mobile phase (A) was prepared with sodium acetate buffer (0.1M, pH 7.2). methanol and tetrahydrofuran were used as an organic modifier (900 : 95 : 5 v/v/v) (Sigma Chemical Co.). Mobile phase (B) was methanol HPLC grade (Merck). The solvents were filtered using filters of 0.22 μm (Millipore Corporation). Proline and hydroxyproline standards (Pierce Chemical Co.) were used. Flow rate was 1.0 mL/min at 25–29°C. The elution gradient of the mobile phase was as follows: 100% A at 0 min; 80% A (0.5–6.5 min); 00% A (8.0–10.0 min) of Cleanup; 100% A (12–15 min) reconditioning. The amino acids were completely eluted at 6 min, and the column was equilibrated for 10 min. The chromatographic conditions and the elution gradient for the mobile phase are presented in Fig. 2. Collagen total content (mg/g) was determined by multiplying the hydroxyproline content by 7.52.[25]

Identification and Quantification

The identification of amino acids was done by using retention time comparison with standards, and by standard addition or "spiking".[26] The detector response for proline and hydroxyproline were calibrated daily, by injecting different amounts of a solution containing 0.01 ng/mL of each standard. The standards were derivatized in the same way as that of the sample. Quantification was done by the use of external standards of proline and hydroxyproline at 0.01 ng/mL (Sigma Chemical Co. A-1879). Calibration factors were calculated from these data. Analyses were carried out in triplicate. All standard solutions and samples were filtered through a 0.22-μm aqueous filter prior to injecting onto the column. Linear regression analysis (Fig. 1) and descriptive statistics were applied (mean, standard deviation, and variation coefficient) (Table 2).

RESULTS AND DISCUSSION

The detector performance was determined because it affects greatly the quantification of proline and hydroxyproline. A sensitivity plot is shown in

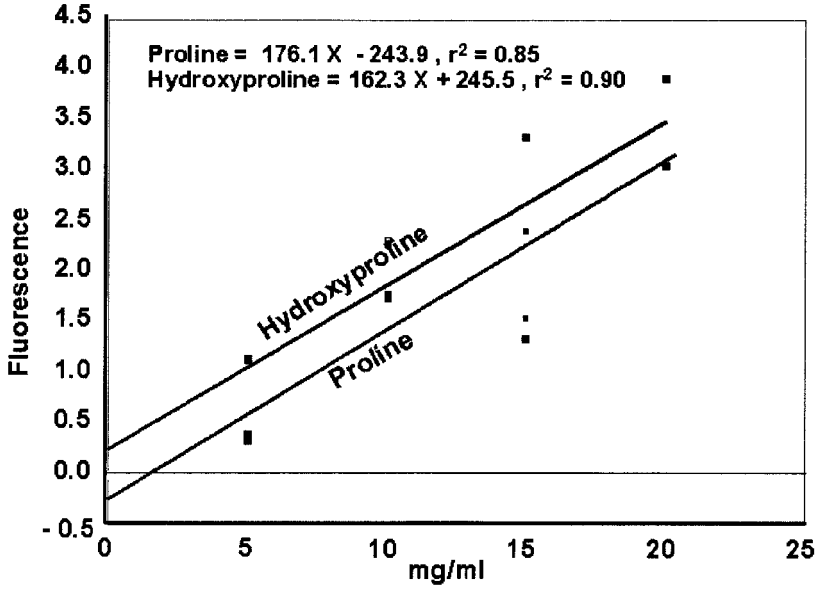

Figure 1. Standard curves exhibiting linearity of peak area vs. mg/mL of proline and hydroxyproline, which were reacted with NBD-Cl.

Fig. 1 for proline and hydroxyproline. The sensitivity for proline and hydroxyproline were 176.1 and 162.3 mV with a linear correlation coefficient of 0.85 and 0.90, respectively, in a range of 0–20 mg/mL. The lower limit of the detector was determined because it is affected by the S/N ratio and the minimal detectable quantity (MDQ = 2 × noise level). The precision of the quantification was S/N ratio = 40 and the MDQ of the detector was 0.0027 ng/mL. The time constant, which controls the electronic filtering used to reduce noise level was set to 1.0 UF (1/3 the peak at half height).

The detector selectivity response of proline and hydroxyproline standards by fluorescence detection system is shown in Fig. 2. Note the baseline noise and the peak size for the fluorescence response. The chromatogram shows a baseline separation. This injection contained approximately 0.01 ng of secondary amino acids. A typical liquid chromatogram of different samples of collagen content is shown in Fig. 3. Of the 14 meat sample tested, only the low collagen samples exhibited interfering peaks with hydroxyproline. However, in these samples the interference was not severe enough to prevent quantification of hydroxyproline. The sample and fluorescence detection system is selective over the matrix sample effect.

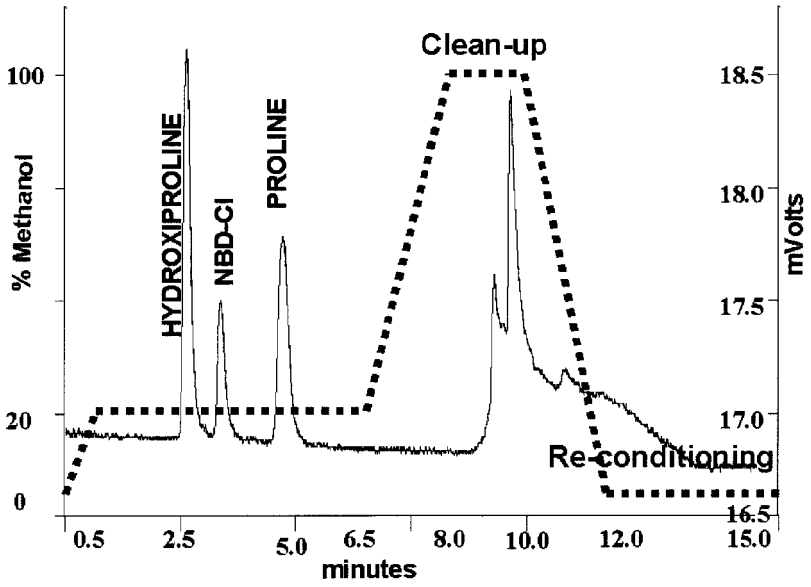

Figure 2. Proline and hydroxyproline standard chromatogram. Mobile phase (A) 0.1 M sodium acetate buffer, pH = 7.2 + methanol + tetrahydrofuran (900 : 95 : 10 v/v/v). Mobile phase (B) methanol pure. Flow rate 1.0 mL/min at 25°C.

The column performance was determined because it mainly affects the resolution and quantification of proline and hydroxyproline. The resolution between two peaks is a function of the retention, selectivity, and column efficiency. In Fig. 2 and Table 1, the column showed good retention and selectivity. The column efficiency showed that the component bands "spread out" in their passage through the column. A more efficient column will give sharper peaks. However, the lower values for height equivalent to theoretical plates (HETP = 0.029 and 0.024) and the high-resolution value, showed a high column efficiency. Particularly, the column used in this report has more than 4000 injections.

A survey of 14 representative meat and meat products samples was conducted. The analytical results are compared with some values reported. In Table 2, the data are average values obtained from triplicate analyses of 3-hydrolized samples. The hydroxyproline results agree quite well with hydroxyproline levels normally found in meat and meat products between 0.05% and 12.5%.[18,23] Analysis of high hyroxyproline samples gave no interfering peaks at the retention times of interest.

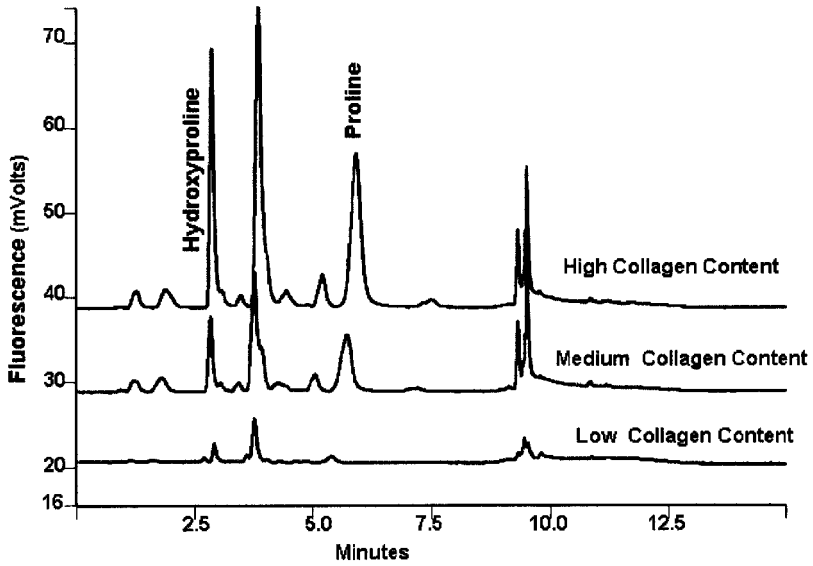

Figure 3. Chromatogram of different levels of hydroxyproline content in samples. High collagen (collagen gel), medium collagen (*Longissimus thoracis*), and low content (bologna 4).

The results reported in Table 2, rating samples by collagen content, indicate collagen gel has the highest collagen content, followed by pig skin, Bologna 7, and Longissimus dorsi. The lean meats and bologna are all in the mid-range and meat and bones have the lowest collagen content.

Collagen has an unusual amino acid composition and sequence. The proportion of glycine residues in all collagen molecules is approximately one-third, which is unusually high for a protein. Every third residue is glycine.

Table 1. Column chromatographic performance.

Chromatographic parameter	Proline	Parameter value	Hydroxyproline
Retention (k')	2.97	1–5	1.22
Selectivity (α)	1.79	>1.5	1.79
Efficiency (N)	338.56	$<10{,}000$	415.14
HETP	0.029	<1.0	0.024
Resolution	3.11	>1.0	3.11

Note: HETP, height equivalent to a theoretical plate.

Table 2. Hydroxyproline, proline, and collagen content in samples.

Product	Hydroxyproline (mg/mL)	Collagen (mg/g)	SD ± CV	Proline (mg/mL)	SD ± CV
Bologna 1	0.46	3.33	0.041 ± 9.00	2.93	0.09 ± 3.08
Bologna 2	0.43	3.12	0.03 ± 7.43	0.67	0.06 ± 9.52
Bologna 3	0.69	5.00	0.007 ± 1.01	0.6	0.05 ± 8.87
Bologna 4	0.39	2.83	0.005 ± 1.22	2.21	0.01 ± 0.60
Bologna 5	0.38	2.80	0.002 ± 0.52	6.34	0.025 ± 0.39
Bologna 6	0.42	3.04	0.019 ± 4.51	4.95	0.12 ± 2.50
Bologna 7	2.23	16.17	0.15 ± 6.75	4.45	0.24 ± 5.44
Longissimus thoracis	1.53	11.09	0.05 ± 3.11	9.59	0.81 ± 8.48
Latissimus dorsi	0.26	1.89	0.009 ± 3.36	2.11	0.093 ± 4.35
Semimembranosus	0.76	5.51	0.03 ± 4.27	28.62	3.22 ± 11.27
Meat and bones	0.06	0.46	0.001 ± 2.41	1.53	0.044 ± 2.86
Fish meal	0.22	1.60	0.01 ± 5.37	0.18	0.26 ± 3.00
Pig skin	5.36	38.86	0.7 ± 13.00	13.21	0.09 ± 0.69
Collagen gel	26.96	195.46	0.44 ± 1.63	37.72	0.29 ± 0.77

Note: SD ± CV, standard deviation ± coefficient of variation.

The amino acid sequence of collagen is remarkably regular: glycine–proline–hydroxyproline recurs frequently.[27] Therefore, a high content of hydroxyproline, as well as a high content of glycine, would indicate a high content of collagen in meat samples. The content of glycine in the samples was analyzed by the method of Vázquez-Ortiz et al.[28] The content of glycine in the samples was high (Table 2).

The proposed HPLC method has been demonstrated to be suitable for analysis of collagen in many commercially available meat and meat products. It represents a considerable improvement over existing methodologies for determination of proline and hydroxyproline, both in analysis time and chemical consumption. The described method can be used in studies that involve collagen as a measure of beef tenderness and changes in intramuscular connective tissue during postmortem time.

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