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Quantitative Analysis of Collagen in Meat Extracts using Liquid Chromatography and Tandem Mass Spectrometry

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Overview

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, including humans. In fact, it makes up about 25% to 35% of the total amount of protein in the body. Hydroxyproline is a major component of the protein collagen playing a key role for collagen's stability. Creatinine is a break-down product of creatine phosphate in muscle. These compounds determine how juicy and tender meat is.

Here we present a method using Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS) for the analysis of hydroxyproline and creatinine from collagen extracts. The samples were simply diluted and injected onto a Hydrophilic Interaction LC column (HILIC) coupled to an API 3200™ LC/MS/MS system operated in positive and negative polarity. Multiple Reaction Monitoring (MRM) was used for detection because of its high selectivity and sensitivity. The developed method had excellent Limits of Detection, linear range and reproducibility and was successfully applied to the analysis of meat extracts.

This analytical procedure can speed up the sample analysis for hydroxyproline and creatinine, which in turn, improves the whole processing of collagen products.

Introduction

Collagen is the main protein of connective tissue in animals and humans. The hydrolysis of collagen results in the formation of gelatin which is used in many food products, dietary supplements, pharmaceutical and cosmetic formulations, and many dental, orthopedic and surgical procedures, such as artificial skin substitutes in the management of severe burns.

Hydroxyproline, a major component of the protein collagen, and creatinine, a break-down product of creatine phosphate in muscle, are measured to determine the juiciness and tenderness of meat. Traditionally, colorimetric methods are used routinely in the meat and leather industries.[1-2]

However, these colorimetric methods require extensive sample preparation, and are subjected to interference with concomitant components in complex tendon extracts. Thus a more accurate and faster analytical method is required.

An LC-MS/MS method was developed to quantify both hydroxyproline and creatinine from meat extracts in one analysis with good sensitivity. The meat extracts were produced by adding hydrochloric acid to tendon in factory concentration tanks. These meat extracts are used to manufacture different meat products to satisfy tastes of consumers, soup flavoring and several meat-based ready-to-serve products.

Experimental

This method was developed using a Shimadzu Prominence LC system interfaced to an AB SCIEX API 3200™ LC/MS/MS system equipped with Turbo V™ source and Electrospray Ionization (ESI) probe. Ion source parameters are listed in Table 1. Targeted analytes were detected in Multiple Reaction Monitoring (MRM). MRM transitions for quantitation and compound identification are listed in Table 2.

LC separation was performed using a GL Sciences Inertsil HILIC column (5 µm) 3 x 150 mm and mobile phase A = acetonitrile + 10 mM ammonium acetate and B = water + 10 mM ammonium acetate (pH 6.7) at a flow rate of 0.5 mL/min. A mobile phase of

(A/B) of 90/10 was used for 4 min and then ramped to 75/25 to 6 min before reequilibration.

Table 1. Ion source parameters using Electrospray Ionization

Parameter	Value
Curtain Gas (CUR)	25 psi
IonSpray Voltage (IS)	5000 V
Temperature (TEM)	500°C
Nebulizer Gas (GS1)	40 psi
Heater Gas (GS2)	60 psi

Table 2. MRM transitions in positive and negative polarity to detect hydroxyproline and creatinine

Analyte	Polarity	Q1	Q3	CE (V)
Hydroxyproline	positive	132.1	86.0	19
	positive	132.1	68.0	25
Hydroxyproline	negative	130.1	84.0	-23
	negative	130.1	82.0	-26
Creatinine	positive	114.1	44.0	27
	positive	114.1	86.0	15
Creatinine	negative	112.1	41.0	-35
	negative	112.1	68.0	-24

Due to high sample acidity (pH 3) the samples were diluted with a mixture of 45 mL acetonitrile, 1.25 mL of 1 M aqueous ammonium acetate solution and 3.75 mL of water. An aliquot of this sample was transferred to 1.7-mL auto-sampler vials for LC-MS/MS analysis.

In addition, the method was verified by analyzing bovine achilles tendon (Sigma-Aldrich, Lot 017K7018). 0.5 g of collagen was digested with a boiling solution of 6 N HCl (62 mL) for 6 hours and filtered through a 2.7-micron glass microfiber. The filtrate was transferred to a volumetric flask, and 6 N HCl was added to bring the total volume to 200 mL. An aliquot of this acidic solution was placed in an auto-sampler vial for LC-MS/MS analysis.

All quantitation data were processed using the MQ II algorithm within Analyst® Software (version 1.5).

Results and Discussion

Hydroxyproline and creatine can be detected in positive and negative polarity using Electrospray Ionization. However, positive polarity offers better sensitivity. An example chromatogram of the analysis of hydroxyproline and creatine in positive polarity is shown in Figure 1 highlighting the superior selectivity and sensitivity of LC-MS/MS operated in MRM.

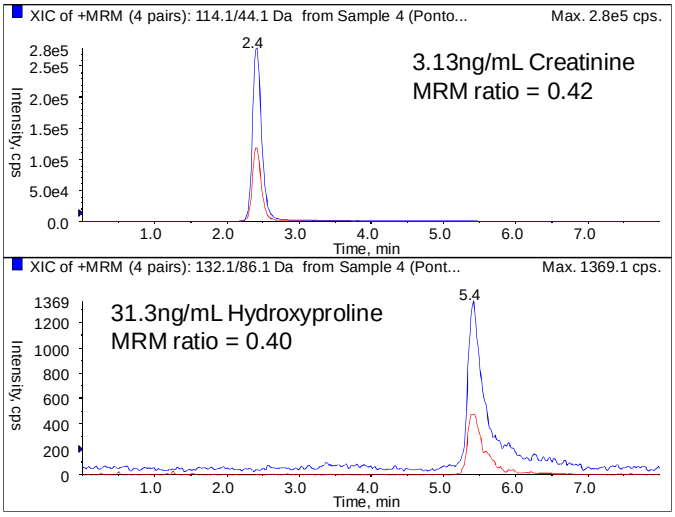

Figure 1. Standard of 3.13ng/mL creatinine and 31.3ng/mL hydroxyproline detected using an API 3200™ LC/MS/MS system

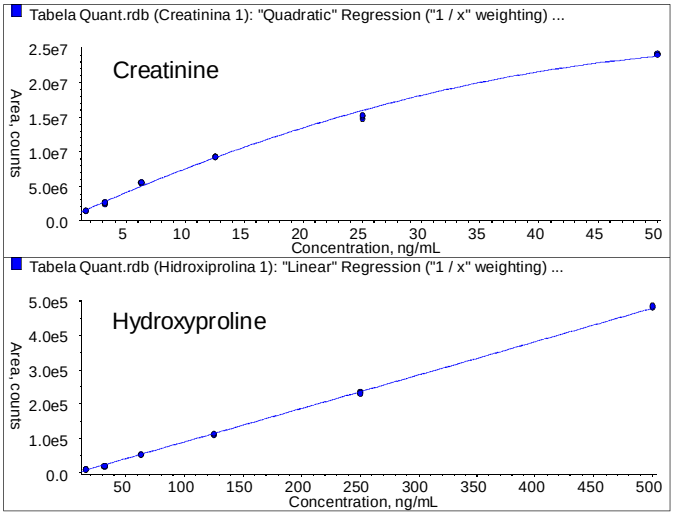

Figure 2. Calibration curves of creatinine (top) hydroxyproline (bottom)

Table 3. Reproducibility (n=3) and accuracy of the quantitation of creatinine and hydroxyproline

Analyte	Sample	Concentration (ng/mL)	Mean (ng/mL)	%CV	Accuracy (%)
Creatinine 1	Standard 6	1.56	1.50	3.2	95.9
	Standard 5	3.13	2.93	4.9	93.6
	Standard 4	6.25	7.10	1.2	113.5
	Standard 3	12.5	12.8	0.8	102.0
	Standard 2	25.0	23.1	2.3	92.5
	Standard 1	50.0	52.5	2.1	104.9
Creatinine 2	Standard 6	1.56	1.50	2.6	96.0
	Standard 5	3.13	2.95	4.1	94.3
	Standard 4	6.25	7.01	1.0	112.2
	Standard 3	12.5	12.8	1.1	102.2
	Standard 2	25.0	23.2	2.3	92.9
	Standard 1	50.0	52.2	4.4	104.4
Hydroxyproline 1	Standard 6	15.6	17.2	2.1	110.2
	Standard 5	31.3	28.3	4.0	90.4
	Standard 4	62.5	63.0	0.8	100.8
	Standard 3	125	123	1.8	98.4
	Standard 2	250	247	0.8	98.9
	Standard 1	500	505	0.6	101.1
Hydroxyproline 2	Standard 6	15.6	17.3	3.1	110.8
	Standard 5	31.3	27.7	1.9	88.5
	Standard 4	62.5	63.7	1.9	101.9
	Standard 3	125	124	1.5	98.8
	Standard 2	250	247	1.2	98.7
	Standard 1	500	505	0.5	101.1

Calibration curves (Figure 2) with excellent accuracies and coefficients of variation (%CV) were obtained in a dynamic range of 1.56 to 50.0 ng/mL for creatinine and 15.6 to 500 ng/mL for hydroxyproline.

Statistical data of both MRM transitions of calibration curves are listed in Table 3. %CV of both analytes over the whole calibration range was <5% with accuracies between 88 and 113%. The high sensitivity of the developed LC-MS/MS method allowed dilution of meat extracts greatly increasing robustness and reducing the risk of possible matrix effects.

Bovine achilles tendon collagen was digested, filtered, diluted, and analyzed by LC-MS/MS to verify method performance (Figure 3).

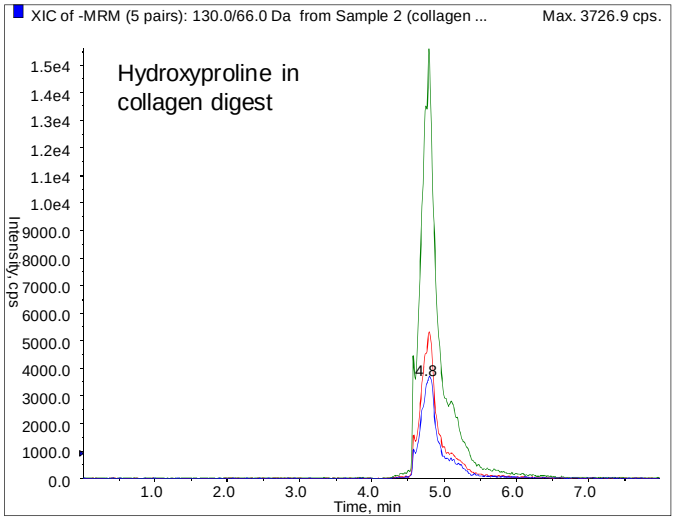

Figure 3. Detection of hydroxyproline in a collagen digest after simple filtration and dilution

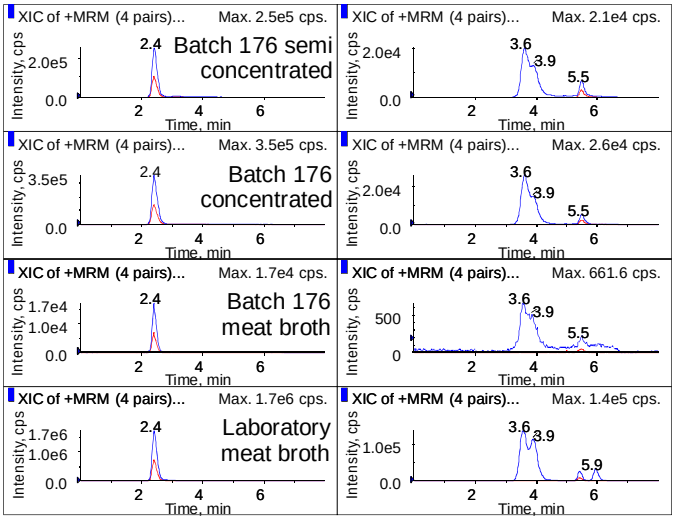

Figure 4. Concentrations of hydroxyproline and creatinine in meat extracts and sample broths, the ratios of quantifier and qualifier MRM transition were used for compound identification with a tolerance of +/- 20% (hydroxyproline 0.32-0.48 and creatinine 0.33-0.50)

Table 4. Concentrations of hydroxyproline and creatinine in meat extracts and sample broths, the ratios of quantifier and qualifier MRM transition were used for compound identification with a tolerance of +/-20% (hydroxyproline 0.32-0.48 and creatinine 0.33-0.50)

	Creatinine		Hydroxyproline	
Sample Name	**Concentration (ng/mL)**	**MRM ratio**	**Concentration (ng/mL)**	**MRM ratio**
Batch 176 semi concentrated meat broth	3.46	0.42	80.1	0.47
Batch 176 concentrated meat broth	5.45	0.41	61.4	0.47
Batch 176 meat broth	< LOD		8.56	0.43
Laboratory meat broth	35.8	0.42	289	0.44

Meat extract samples were analyzed with good selectivity and sensitivity. These extracts were sampled from factory tanks, which are used at the start of the meat extract concentration process. The samples were simply diluted and injected into the LC-MS/MS system, without any additional extraction or clean-up process (Figure 4). The concentrations of hydroxyproline and creatine ranging from 8.6 ng/mL to 289 ng/mL and 3.5 ng/mL to 36 ng/mL from, respectively, are listed in Table 4. The quantifier MRM transition was used to determine the concentration of the targeted analytes in the unknown samples. In addition, the ratio of quantifier and qualifier MRM transition was used to further identify hydroxyproline and creatinine. The MRM ratio of unknown samples was compared to an average of all standard injections with a tolerance of +/- 20%.

Summary

We demonstrated that it is possible to detect and quantify hydroxyproline and creatinine in meat extracts with good detection and quantitation limits within an 8 min chromatographic run.

This LC-MS/MS method can replace the traditional colorimetric method used in the meat and leather industry. This method offers faster analysis time and more accurate data compared to the colorimetric method.

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