Application Note

053/2010

SpeedDigester K-436, K-439

Determination of Hydroxyproline in Meat after Acid Hydrolyzation (Photometric method)

SHORT NOTE

Determination of Hydroxyproline in Meat after Acid Hydrolyzation (Photometric method)

An easy procedure for hydroxyproline determination in meat products according to the photometric method, as described in the LFBG §64 06.00-8 and ISO 3496:1994, is introduced below. The samples undergo an acid hydrolysis with hydrochloric acid using the SpeedDigester K-436 or K-439, followed by a photometrical determination at 558 nm. There are no differences between the results obtained with the K-436 and the K-439 respectively. The determined hydroxyproline contents are reliable and reproducible and correspond to the certified values.

Introduction

The determination of hydroxyproline in meat products is an often used parameter for the evaluation of meat quality. Hydroxyproline is a part of collagen and occurs therefore only in sinews, bones, gristle and skin. A high amount of hydroxyproline is an indication that raw material with cheap quality was used. The samples require acid hydrolyzation to set the hydroxyproline free from the connective tissue and separated from fat. With chloramine T the hydroxyproline will be oxidized to pyrrol. This compound will be converted to a colored product with 4-dimethylaminobenzaldehyde and afterwards determined with a photometer at 558 nm.

Experimental

Instrumentation: SpeedDigester K-436, K-439 with Reflux Digestion Set

Samples: Processed meat
(certified value: 0.356 ± 0.004 g/100 g)

Preparation of the sample solutions:

2.0 g of the homogenized sample were added directly into a sample tube. A portion of 30 ml of hydrochloric acid 6 mol/l and a few anti bumping granules were added. The hydrolisation was performed using the parameters specified in Table 1.

Table 1: Temperature profile for hydrolisation with the K-436, K-439

	K-439		K-436	
Step	Temp. [°C]	Time [min]	Level	Time [min]
Preheat	300	-	5.0	10
1	300	480	5.0	480
Cooling	-	30	-	30

The samples were cooled down to ambient temperature and transferred with water into a 100 ml volumetric flask. 3-5 ml petroleum ether were added and the samples were shaken strongly. The volumetric flask was filled up with water to the calibration mark so that the petroleum layer was above the mark. The sample was shaken strongly again and the layers were let separated. The petroleum ether layer was removed. The water layer was filtered through a filter paper into a conical flask.

Preparation of the standard solutions:

Stock solution:
120 mg hydroxyproline were weighed in a 200 ml volumetric flask and filled up with water to the calibration mark.

Standard solutions:
Prepared from stock solution with concentrations between 1.2 – 8.4 µg/100 µl.

Chloramine T reagent:
Buffered Chloramine T solution for oxidation the hydroxyproline.

Color reagent:
Mixture of 4-Dimethylaminobenzaldehyde in perchloric acid and 2-propanol.

Preparation of the measuring solutions:

The preparations of the sample-, blank- and standard solutions were made in test tubes and are described in Table 2.

Table 2: Preparation of the measuring solutions

	Sample solution	Standard solution	Blank solution
Sample filtrate	100 µl	-	-
Standard solution	-	100 µl	-
Water	-	-	100 µl
Chloramine T reagent	5 ml	5 ml	5 ml
Let the test tubes stand for about 20 min at ambient room temperature			
Color reagent	2 ml	2 ml	2 ml
Close the test tubes, shake vigorously and let them stand in a water bath with 60 ±0.5°C for 15 min. Cool down to ambient temperature and let them stand for 30 min. Measure the absorbance at 558 nm against the blank. The sample amount is calculated taking the regression line into account.			

Results

The determined hydroxyproline contents are presented in Table 3.

Table 3: Determined hydroxyproline contents in processed meat (relative standard deviation in brackets, n=6, certified value 0.356 g/100g)

	Hydroxyproline K-439 [g/100 g]	Hydroxyproline K-436 [g/100 g]
Processed meat	0.356 (2.36 %)	0.356 (2.86 %)

Conclusion

The determination of hydroxyproline in processed meat according to LFBG §64 6.00-8 and ISO 3496:1994 using SpeedDigester K-436 or K-439 provides reliable and reproducible results which correspond to the certified value.

References

LFBG §64 06.00-8
ISO 3496:1994
Operation manual SpeedDigester K-425 / K-436
Operation manual SpeedDigester K-439

For more detailed information please refer to Application Note 053/2010

1 Introduction

The determination of hydroxyproline in meat products is an often used parameter for the evaluation of meat quality. Hydroxyproline is a part of collagen and occurs therefore only in sinews, bones, gristle and skin. A high amount of hydroxyproline is an indication that raw material with cheap quality was used.
An easy and reliable method for the determination of hydroxyproline in processed meat, as described in the LFBG §64 06.00-8 and ISO 3496:1994, is introduced below. The samples require acid hydrolyzation to set the hydroxyproline free from the connective tissue and separated from fat. With chloramine T the hydroxyproline will be oxidized to pyrrol. This compound will be converted to a colored product with 4-dimethylaminobenzaldehyde and afterwards determined with a photometer at 558 nm (reaction equation in Figure 1).

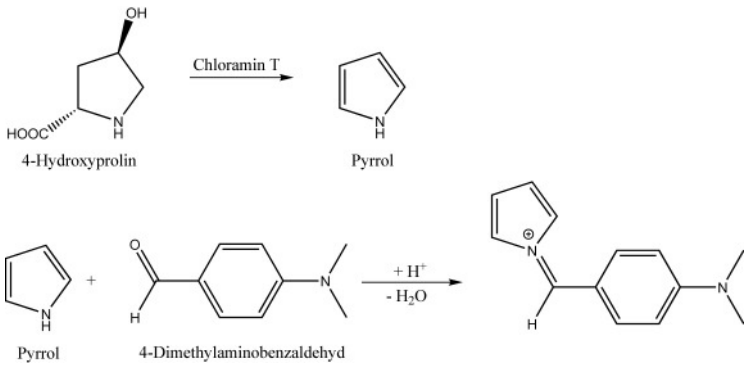

Figure 1: Reaction for the determination of hydroxyproline

2 Equipment

- SpeedDigester K-436, K-439 (the parameters used for K-436 are also valid for Büchi SpeedDigester K-425)
- Reflux Digestion Set (Büchi No. 11056853)
  with glass caps for COD (6 x Büchi No. 011220)
- Photometer Helios Delta (Fa. Thermo) 558 nm
- Thermostatic water bath to 60°C
- Analytical balance (accuracy ± 0.1 mg)
- Mixer B-400

3 Chemicals and Materials

- Chloramine T trihydrate, Merck (1.02426)
- 4-Dimethylaminobenzaldehyde, Merck (8.03057)
- L-Hydroxyproline, Merck (1.04506)
- Hydrochloric acid 6 mol/l
  295 ml hydrochloric acid 32%, Hänseler (20-2000-5) are filled up to 500 ml with deionised water

- Petroleum ether boiling range 40-60°C, Scharlau (ET0093005M)
- Citric acid monohydrate, Fluka (27490)
- Sodium hydroxide pellets, Merck (1.06495)
- Sodium acetate anhydrous, Fluka (71183)
- Perchloric acid 60%, Fluka (77232)
- 1-Propanol, Merck (1.00996)

- 2-Propanol, Fluka (59300)
- Buffer solution pH 6.8

  26.0 g citric acid monohydrate, 14.0 g sodium hydroxide pellets and 78.0 g sodium acetate anhydrous are weighed in a 1000 ml volumetric flask and dissolved in 500 ml deionised water. 250 ml 1-propanol were added and filled up with deionized water to the calibration mark.

- Hydroxyproline stock solution (120.0 mg / 200.0 mL)

  Approx. 120 mg hydroxyproline are weighed in a 200 ml volumetric flask, dissolved in deionised water and filled up with water to the calibration mark.

- Hydroxyproline standard solutions

  Prepared from stock solution as described in Table 1

Table 1: Pipetting scheme for preparing the standard solutions

	Hydroxyproline standard solutions						
	1	2	3	4	5	6	7
Hydroxyproline stock solution	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	7 ml
Dilute with water to	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml	50 ml
Amount [µg/100 µl]	1.2	2.4	3.6	4.8	6.0	7.2	8.4

- Oxidation reagent

  1.4 g chloramine T are dissolved in 100 ml buffer solution pH 6.8 and diluted with 100 ml deionised water.

- Color reagent

  10.0 g 4-dimethylaminobenzaldehyde are dissolved in 35 ml perchloric acid 60% and diluted with 65 ml 2-propanol.

- 100 ml volumetric flask with min. 8 ml air space above the mark
- Folded filters 595 ½, Ø 150 mm, Schleicher & Schuell (10311 645)
- Disposable cuvettes 1 cm, Brand (7591 05)
- Anti bumping granules, Fluka (85106)
- Closable test tubes with 15 – 20 ml volume

4 Sample

- Processed meat (certified reference material from Lvu, Germany)
  Amount of hydroxyproline: 0.356 ± 0.004 g/100 g

The sample was homogenized with the B-400 for 2 s up to visual homogeneity.

5 Procedure

The determination of hydroxyproline in meat includes the following steps:

- Homogenization of the sample
- Acid hydrolysis of the sample, using SpeedDigester K-436, K-439
- Separation the sample from the fat
- Oxidation of the hydroxyproline
- Measuring the absorbance at 558 nm
- Calculation the amount of hydroxyproline

5.1 Acid hydrolysis

- Place approx. 2.0 g of the homogenized sample in a 300 ml sample tube
- Add 30 ml hydrochloric acid 6 mol/l and a few anti bumping granules
- Carefully suspend the sample by gently swirling the tube
- Place the sample tubes into the Reflux Digestion Set and fix each sample tube with a clamp
- Insert the Reflux Digestion Set containing the samples into the preheated unit and turn on the cooling water
- Digest the samples according to the parameters listed in Table 2

Table 2: Temperature profile for hydrolization with the K-436, K-439

	K-439		K-436	
Step	Temperature [°C]	Time [min]	Heating Level	Time [min]
Preheating	300	-	5.0	10
1	300	480	5.0	480
Cooling	-	30	-	30

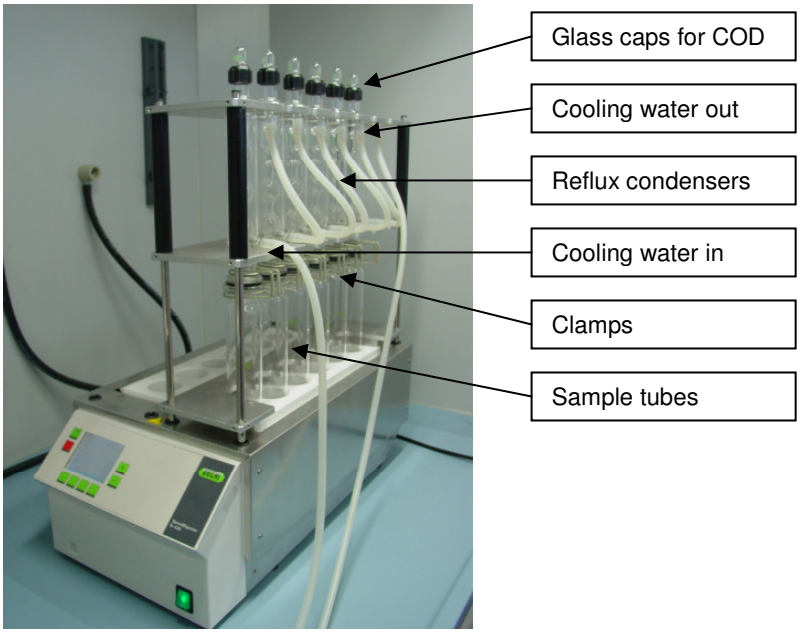

Figure 2: SpeedDigester K-439 with Reflux Digestion Set

NOTE: When the Reflux Digestion Set is placed outside the SpeedDigester it takes approx. 30 min to cool them down; when they are left in the heating chamber it takes at least 60 min.

- Let the samples cool down to ambient temperature
- Transfer the sample into a 100 ml volumetric flask and rinse sample tube with deionized water
- Add 3-5 ml petroleum ether and shake strongly
- Fill up with deionized water to the calibration mark so that the petroleum ether layer is above the mark
- Shake again strongly and let the layer separate
- Aspirate the petroleum ether layer with e.g. a water jet pump
- Filtrate the water layer through a filter paper into a conical flask

NOTE: Connect the extraction hose from the water jet pump with a pasteur pipette and aspirate the petroleum layer. You can also use a pipette to remove the layer.

5.2 Preparation of the measuring solutions

The preparations of the sample-, blank- and standard solutions were made in test tubes and are described in Table 3.

Table 3: Preparation of the measuring solutions

	Sample solution	Standard solution	Blank solution
Sample filtrate	100 µl	-	-
Standard solution	-	100 µl	-
Water	-	-	100 µl
Oxidation reagent	5 ml	5 ml	5 ml
Let the test tubes stand for about 20 min at ambient room temperature			
Color reagent	2 ml	2 ml	2 ml
Close the test tubes, shake vigorously and let them stand in a water bath with 60 ±0.5°C for 15 min. Cool down within 3 min to ambient temperature e.g. under cold water and let them stand for 30 min.			
Measure the absorbance at 558 nm against the blank			

5.3 Calculation

5.3.1 Calculation regression line

The regression line is calculated taking into account the measured values from the standard solutions. The formula (1) is used to calculate the slope and the axis intercept from the regression line.

$$y = ax + b \qquad (1)$$

y : absorbance standard solution at 558 nm

a : slope

x : amount hydroxyproline standard solution [µg/100 µl]

b : axis intercept

NOTE: The calculation of the regression line and the amount of hydroxyproline in the samples were done with the usage of Excel.

5.3.2 Calculation amount hydroxyproline in the sample

The amount of hydroxyproline in the sample is calculated using the following equations (2) – (3)

$$w = \frac{(y - b) \cdot 100 \cdot 100}{a \cdot m_{Sample} \cdot 0.1 \cdot 1'000'000} \qquad (2)$$

Simplified calculation:

$$w = \frac{(y - b)}{a \cdot m_{Sample} \cdot 10} \qquad (3)$$

w	: weight fraction of hydroxyproline
y	: absorbance sample solution at 558 nm
a	: slope from regression line
b	: axis intercept from regression line
m_{Sample}	: sample weight [g]
100	: conversion factor [%]
100	: volume of volumetric flask [ml]
0.1	: volume sample filtrate [ml]
1'000'000	: conversion factor [µg/g]

6 Results

6.1 Regression line

The amount of hydroxyproline and the measured absorbance is listed in table 4. Figure 2 shows the regression line with the calculated formula and regression coefficient.

Table 4: Amount and absorbance of the standard solutions (Concentration of the stock solution: 122 mg/200 ml)

	Hydroxyproline standard solutions						
	1	2	3	4	5	6	7
Amount [µg/100 µl]	1.22	2.44	3.66	4.88	6.10	7.32	8.54
Absorbance	0.067	0.143	0.220	0.297	0.362	0.435	0.503

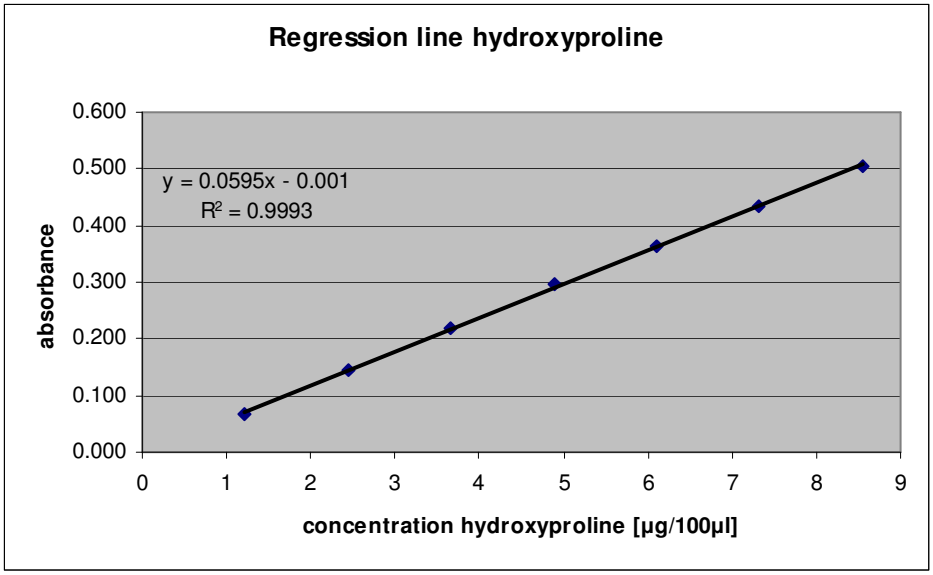

Figure 2: Regression line of hydroxyproline

6.2 Digestion with SpeedDigester K-439

The results of the determination of hydroxyproline in processed meat are presented in Table 5.

Table 5: Results for the determination of hydroxyproline in processed meat with K-439

Processed meat	m_{Sample} [g]	Absorbance	Amount hydroxyproline [g/100 g]
Sample 1	2.0643	0.430	0.351
Sample 2	2.0473	0.441	0.363
Sample 3	2.2059	0.477	0.364
Sample 4	2.0668	0.441	0.359
Sample 5	2.1070	0.446	0.356
Sample 6	2.1145	0.429	0.342
Average	-	-	**0.356**
Rsd [%]	-	-	**2.36**

6.3 Digestion with SpeedDigester K-436

The results of the determination of hydroxyproline in processed meat are presented in Table 6.

Table 6: Results for the determination of hydroxyproline in processed meat with K-436

Processed meat	m_{Sample} [g]	Absorbance	Amount hydroxyproline [g/100 g]
Sample 1	2.3507	0.472	0.338
Sample 2	1.9600	0.415	0.356
Sample 3	2.1336	0.460	0.363
Sample 4	1.9755	0.415	0.354
Sample 5	2.1142	0.462	0.368
Sample 6	2.2657	0.481	0.357
Average	-	-	**0.356**
Rsd [%]	-	-	**2.86**

7 Comparison to Standard Method

This application note is based on the standard methods LFBG 06.00-8 and ISO 3496:1994

Table 7: Differentiation to the standard methods

	This application note	LFBG 06.00-8	ISO 3196:1994	Notes/Impact
Heating source	SpeedDigester K-436 or K-439	Heating plate	Drying oven 105°C ±1 °C	No impact. The usage of the SpeedDigester is more comfortable.
Digestion flask	Reflux Digestion Set. 300 ml sample tubes	Erlenmeyer flask or round bottom flask with condenser	200 ml round- or flat-bottom flask with watch glas	No impact. The Reflux Digestion Set is easy to handle.
Chloramine T reagent	1.4 g chloramine T diluted in 100 ml buffer solution and 100 ml water	1.4 g chloramine T diluted in 100 ml buffer solution and 100 ml water	1.4 g chloramine T diluted in 100 ml buffer solution	No impact. It is important that the hydroxyproline is completely oxidated.
Acid hydrolysation	Hydrochloric acid 6 mol/l	Hydrochloric acid 6 mol/l	Sulfuric acid 3 mol/l	No impact. Same concentration of H_3O^+.
Stock solution	120 mg hydroxyproline in 200 ml water	120 mg hydroxyproline in 200 ml water	50 mg hydroxyproline in 100 ml water	No impact. Approx. same concentration of hydroxyproline.
Standard solutions	Concentrations between 1.2 – 8.4 µg/100 µl (= 12 – 84 µg/ml)	Concentrations between 1.2 – 8.4 µg/100 µl (= 12 – 84 µg/ml)	Concentrations between 0.5 - 2 µg/ml	No impact. Same concentration in cuvette because of used volume for measuring solution. -LFBG: 1.2 – 8.4 µg/cuvette - ISO: 2 – 8 µg/cuvette
Test portion	2 g	2 g	4 g	No impact. Approx. same ratio of sample/volume
Volumetric flask	100 ml	100 ml	250 ml	
Hydrolysis time	8 h	8 h	16 h	No impact. It is important that the sample is completely digested. 8 h are sufficient.
Defatting of the sample solution	Yes	Yes	No	The sample solution was defatted with petroleum ether.
Sample volume	100 µl	100 µl	4ml	No impact. It is important that the samples and the standard solutions are measured with the same method. The amount of hydroxyproline has to be in the range of the regression line.
Reaction time at 60°C	15 min	15 min	20 min	

8 Conclusion

The determination of hydroxyproline in processed meat using the SpeedDigester K-436 or K-439 provides reliable and reproducible results that correspond to the certified value. There are no differences between the results obtained with the K-436 and the K-439 respectively.

9 References

LFBG § 64 L06.00-8
ISO 3496:1994

Operation manual of SpeedDigester K-425 / K-436
Operation manual of SpeedDigester K-439
Operation manual of Helios Delta (Thermo, USA)

BÜCHI Labortechnik AG
CH-9230 Flawil 1/Switzerland
T +41 71 394 63 63
F +41 71 394 65 65
www.buchi.com

Quality in your hands