Spectrophotometric determination of hydroxyproline (collagen) content in meat products. Comparative study according to sample preparation.

DEWEGHE, L., HEREMANS, F., LENGES, J.
Analytical and Experimental Station, C.O.O.V.I.-C.E.R.I.A., 1070 Brussels, Belgium.

I. Introduction

The assessment of collagen in meat products is important for the following two reasons :

1. Collagen is considered to be of low nutritional value : the total essential amino-acid content is significantly lower than for meat proteins and there is a complete lack of the essential amino-acid tryptophan.
2. Belgian legislation has set a maximum value for the collagen/protein ratio for a series of meat products.

Hence, the need for a reliable analytical method to assess the collagen content. As a rule, the assessment of collagen is carried out by means of a spectrophotometric determination of the amount of hydroxyprolin occuring in the sample after hydrolysis in acid medium.

The ISO-method, derived from the STEGEMANN and STALDER method, is frequently used, or else an alternative simplified method, which has been set up by the "Arbeitsgruppe Fleischwaren des Arbeitskreises Nordrhein-Westfalen in der GDCh - Fachgruppe Lebensmittelchemie und gerichtliche Chemie" (1970), is applied. A comparative study, carried out in our laboratory (1972) showed that the alternative method is suitable for routine controls. The main asset of the alternative procedure as compared to the ISO-method is the gain in time and in energy costs (the time of hydrolysis is reduced from 16 h to 7 h).

In practice, however, problems do occur sometimes as e.g. during hydrolysis, and this entails poor reproducibility of the results and low percentage of recovery.

Because of this, some analysts carry out a preliminary fat extraction of the sample (a method has been set up by the "Onderzoekscentrum voor Voeding, Veeteelt en Vleestechnologie" of the Faculty of Agricultural Sciences, R.U.G.

The other main differences as compared to the simplified ISO-method are :

- composition of the reagent for the hydrolysis
- nitrogen flushing prior to hydrolysis
- duration of the hydrolysis
- ion exchange treatment prior to the colorimetric reaction

The extra manipulations increase considerably the time as well as the cost of the analysis. In the present study the simplified ISO-method (method 1) has been compared to the method of the "Onderzoekscentrum voor Voeding, Veeteelt en Vleestechnologie, R.U.G." (method 2).

II. Methods

Principle :

Both methods are based on the same principle :
The sample is first hydrolysed in acid medium to liberate hydroxyprolin from the collagen. Then the hydrolysat is oxidized with chloramin-T. The oxidized hydroxyprolin is measured by colorimetry using p-dimethylamino-benzaldehyde.

The detailed procedures are given in the references 1 (method 1) and 6 (method 2).
The table hereunder compares the different analytical steps of both methods, pointing out the main differences.

Table 1 : Different analytical steps and main differences of both methods

	Method 1	Method 2
Sample size	7-8 g	5 g
Fat extraction	-	10 times with petroleum ether
Hydrolysis		
- time	7 h	24 h
- reagent	30 ml solution : 41.7 g $SnCl_2.2\ H_2O$ dissolved in 280 ml H_2O + 700 ml HCl (d = 1,19)	25 ml HCl 6N
- gas blanketing	-	N_2
- ion exchange	-	mixture of activated charcoal and Dowex 1 (Fluka) (1/2)
Filtration	on S&S 589[2]	not specified
Neutralizing	-	neutralizing with NaOH or HCl for pH 5-9
Oxidation	2 ml filtered, if necessary diluted hydrolysat + 1 ml chloramin-T buffer	10 ml filtered, if necessary diluted hydrolysat + 5 ml chloramin-T buffer
	Composition of oxidant :	Composition of oxidant :
	10 ml solution 1 + 90 ml solution 2	1.41 g chloramin-T + 10 ml H_2O + 80 ml buffer solution
	solution 1 : dissolve 14.1 g chloramin-T in 100 ml H_2O	Buffer solution : dissolve 50 g citric acid monohydrate + 12 ml acetic acid glacial + 120 g sodium acetate trihydrate + 34 g sodium hydroxide in 800 ml H_2O; bring to pH 6 with sodium hydroxide or citric acid; make up to volume 1 litre,
	solution 2 : buffer : dissolve 30 g citric acid monohydrate + 15 g sodium hydroxide + 90 g sodium acetate trihydrate in 500 ml H_2O	

	Method 1	Method 2
	+ 290 ml n-propanol + 5 drops toluene; make up to volume 1 litre.	add 200 ml H_2O + 300 ml n-propanol.
	Leave to react at room temperature for 20 minutes.	Leave to react at room temperature for 20 minutes.
Colorimetry	+ 1 ml colorimetric reagent	+ 5 ml colorimetric reagent
	Composition of colorimetric reagent :	Composition of colorimetric reagent :
	15 g p-dimethylaminobenzaldehyde + 26 ml perchloric acid 60 % + 62 ml n-propanol	10 g p-dimethylaminobenzaldehyde + 35 ml perchloric acid 60 % + 65 ml iso-propanol
	15 min at 60 °C	15 min at 60 °C
Assessment	absorbance at 558 nm	absorbance at 560 nm

III. Field of application - Results obtained

A total of 42 samples, including 16 raw materials and 26 meat products, have been analyzed in duplicate by both methods. For each analysis the recovery was measured after standard addition of gelatin. Table 2 shows the results of all samples for the hydroxyprolin contents obtained by the first and by the second determination, the mean hydroxyprolin content, the % of recovery after addition of gelatin and the mean collagen content, as well for method 1 as for method 2.

Both methods have been adapted for a low fat containing meat product (cooked sausage - saucisson de Paris, fat content 10.2 %) as well as for a high fat containing meat product (liver pie - pâté de foie, fat content 24.5 %).

The results obtained for a series of 10 duplicate analyses are shown in table 3.

Table 2 : Analytical results for 42 samples according to methods 1 and 2

Serial Number	Product	Method 1					Method 2				
		% Hydroxyprolin			% Collagen	% recovery	% Hydroxyprolin			% Collagen	% recovery
		1	2	Mean			1	2	Mean		
1	Beef	.44	.46	.45	3.63	86.7	.36	.40	.38	3.05	98.6
2 (1)	Beef	.37	.38	.38	3.01	101.2	.41	.44	.42	3.42	103.2
3 (1)	Beef	.28	.29	.28	2.26	101.7	.31	.30	.30	2.42	96.9
4 (1)	Beef	.36	.39	.38	3.01	107.9	.42	.40	.41	3.28	100.7
5	Beef	.31	.35	.33	2.64	109.8	.35	.36	.35	2.81	102.2
6 (1)	Beef	.36	.39	.37	2.99	106.3	.33	.35	.34	2.70	105.4
7	Beef	.37	.34	.35	2.82	124.8	.35	.37	.36	2.88	97.7
8 (1)	Beef	.39	.40	.39	3.16	110.1	.39	.36	.38	3.01	102.6
9 (1)	Beef	.33	.30	.32	2.52	102.1	.33	.30	.32	2.51	103.8
10 (1)	Horse meat	.32	.31	.32	2.50	108.5	.37	.34	.35	2.83	98.3
11 (1)	Horse meat	.35	.35	.35	2.82	85.8	.31	.34	.33	2.60	97.4
12 (2)	Pork	.21	.21	.21	1.68	104.3	.23	.21	.22	1.76	108.0
13	Pork	.20	.18	.19	1.52	96.4	.18	.18	.18	1.45	104.8
14	Pork	.20	.22	.21	1.68	112.6	.24	.25	.24	1.94	101.1
15	Pork	.25	.23	.24	1.92	109.9	.26	.29	.27	2.20	104.2
16	Pork	.22	.21	.21	1.70	105.4	.24	.22	.23	1.85	97.7
17	Dry sausage	.27	.27	.27	2.14	100.9	.24	.22	.23	1.87	98.0
18	Dry sausage	.46	.45	.46	3.62	101.4	.44	.46	.45	3.59	95.2
19	Dry sausage	.52	.52	.52	4.13	103.5	.47	.43	.45	3.62	94.8
20	Dry sausage	.49	.46	.47	3.78	108.4	.46	.46	.46	3.66	94.9
21	Dry sausage	.52	.52	.52	4.17	95.3	.48	.50	.49	3.92	76.9
22	Dry sausage	.49	.47	.48	3.84	96.3	.47	.46	.47	3.73	93.8
23 (2)	Dry sausage	.35	.34	.34	2.75	97.3	.29	.30	.29	2.35	102.6
24 (2)	Dry sausage	.44	.44	.44	3.52	98.1	.46	.43	.44	3.56	97.5
25 (2)	Dry sausage	.50	.53	.52	4.12	105.2	.48	.48	.48	3.82	93.8
26 (2)	Dry sausage	.48	.47	.48	3.83	97.4	.51	.51	.51	4.07	92.4
27 (2)	Dry sausage	.39	.39	.39	3.12	98.8	.41	.40	.40	3.24	75.7
28 (2)	Dry sausage	.40	.41	.40	3.24	103.3	.39	.40	.40	3.16	-
29 (2)	Dry sausage	.46	.44	.45	3.60	99.6	.45	.45	.45	3.58	95.5

Serial Number	Product	Method 1					Method 2				
		% Hydroxyprolin			% Collagen	% recovery	% Hydroxyprolin			% Collagen	% recovery
		1	2	Mean			1	2	Mean		
30	Liver pie	.28	.26	.27	2.16	95.4	.24	.24	.24	1.92	98.2
31	Liver pie	.22	.24	.23	1.84	100.0	.23	.21	.22	1.76	96.3
32 (2)	Liver pie	.25	.24	.24	1.94	98.7	.25	.25	.25	1.97	94.7
33 (2)	Liver pie	.26	.23	.24	1.95	99.7	.24	.23	.24	1.90	96.4
34 (2)	Liver pie	.25	.25	.25	1.98	98.5	.21	.24	.22	1.77	99.0
35 (2)	Liver pie	.25	.24	.24	1.96	-	.24	.25	.24	1.94	98.3
36 (2)	Liver pie	.25	.24	.25	1.97	99.3	.24	.24	.24	1.94	96.9
37 (2)	Liver pie	.26	.25	.25	2.03	93.0	.25	.25	.25	2.00	95.9
38 (2)	Liver pie	.24	.24	.24	1.96	96.8	.22	.23	.23	1.80	101.4
39	Liver pie	.24	.24	.24	1.92	92.3	.23	.23	.23	1.81	82.6
40	Liver pie	.23	.23	.23	1.86	97.8	.22	.23	.23	1.80	96.4
41	Liver pie	.25	.24	.24	1.96	100.1	.24	.21	.23	1.81	99.3
42	Liver pie	.24	.24	.24	1.91	105.8	.22	.24	.23	1.85	97.6

(1) : products with fat content below 10 %

(2) : products with fat content above 20 %

Table 3 : Results obtained for 10 determinations of hydroxyprolin in cooked sausage and in liver pie, according to both methods

	Method 1		Method 2	
	% Hydroxyprolin	% Collagen	% Hydroxyprolin	% Collagen
Cooked sausage (fat content = 10.2 %)	.18	1.48	.13	1.01
	.17	1.34	.13	1.05
	.18	1.46	.14	1.13
	.18	1.43	.12	1.00
	.17	1.37	.13	1.07
	.16	1.32	.12	1.00
	.16	1.26	.14	1.14
	.17	1.33	.14	1.14
	.18	1.48	.12	0.99
	.15	1.23	.13	1.06
Mean	.17	1.37	.13	1.06
Liver pie (fat content = 24.5 %)	.32	2.56	.31	2.49
	.31	2.48	.29	2.31
	.29	2.33	.30	2.44
	.32	2.54	.29	2.29
	.32	2.59	.30	2.40
	.29	2.34	.30	2.39
	.33	2.65	.27	2.20
	.34	2.71	.27	2.20
	.34	2.72	.30	2.42
	.32	2.60	-	-
Mean	.32	2.55	.29	2.35

IV. Discussion of results and conclusions

1. The % recovery assessed after standard addition for 42 samples is :
   - for method 1 : between 85.8 and 124.8 %, with a mean value of 101.4 % and a variance coefficient of 6.6 %;
   - for method 2 : between 75.7 and 108.0 %, with a mean value of 97.2 % and a variance coefficient of 7.2 %.

   For the raw materials as such (16 out of 42 samples) the % recovery is noticeably better for method 2 than for method 1 (96.9 - 108.0, mean = 101.4 % as compared to 85.8 - 124.8, mean = 104.6 %).

2. The correlation coefficient between both methods, computed for 42 samples is 0.97.
   According to the t-test and to the WILCOXON test, no significant difference exists between the two methods.

3. For 8 samples with low fat content (raw material with fat content below 10 % - see table 2), the correlation coefficient between the two methods is 0.69. (It is noteworthy that the results are rather grouped).
   According to the t-test and to the WILCOXON test, no significant difference was found between method 1 and 2.

4. For 15 samples with high fat content (meat products with fat content above 20 %) the correlation coefficient between the two methods is 0.98.
   Neither the t-test, nor the WILCOXON test shows a significant difference between both methods.

5. Ten analyses of a sample of cooked sausage (fat content 10.2 %) and 10 analyses of a sample of liver pie (fat content 24.5 %) give the following results :

	mean hydroxyprolin content (%)		mean collagen content (%)	
	method 1	method 2	method 1	method 2
cooked sausage	0.17 ± 0.01	0.13 ± 0.01	1.37 ± 0.06	1.06 ± 0.04
liver pie	0.32 ± 0.02	0.29 ± 0.01	2.55 ± 0.10	2.35 ± 0.08

   As for the repeatability of the analyses, method 2 gives less scattering of the results than method 1.
   The t-test shows a significant difference between both methods for the cooked sausage as well as for the liver pie; the difference is rather less significant for the liver pie.

Based on the analytical results of the 42 samples it appears that the simplified ISO-method (method 1) is suitable for routine analyses.
A preliminary fat extraction for high fat containing samples does not seem to influence the results in a significant way.
In our opinion, the homogenization of the sample to be analyzed is of great importance.

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