

STATISTICAL EVALUATION OF THE RESULTS OBTAINED WITH THE ISO-METHOD FOR HYDROXY-
PROLIN ASSAY (STEGEMANN-STALDER) - INFLUENCE OF SOME MODIFICATIONS.

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I. INTRODUCTION.

In the scope of round robin tests carried out for ISO (International Organization for standardization) TC 34/SC 6/WG 2 - sampling and testing methods of meat and meat products, a collaborative comparative study of the four methods proposed by ISO has been made by the Group members (11, 12, 13, 14)

These methods are the following :

- 1) Möhler K. and Antonacopoulos N.
- 2) Bergman I. and Loxley R.
- 3) Stegemann H. and Stalder K.
- 4) Stegemann-Stalder method modified by Huryck-Ghvapil and Möhler-Volley

The fundamentals of these four methods are summarized hereunder :

1. MOHLER K. and ANTONACOPOULOS N. : (10a, 16)

The minced homogenized material is hydrolyzed by reflux boiling (for 7 h) with HCl 6N added with stanneous chloride (II) (to avoid the formation of humines). After neutralization and oxidation of Hydroxyproline (HP) by Hydrogen peroxide added with Copper sulphate, the oxidation product reacts with 4-dimethyl-amino-benzaldehyde. The red colour is then evaluated by spectrophotometric measurement at 560 nm (ISO 136)

2. BERGMAN I. and LOXLEY R. (7, 10b)

Hydrolysis of the test sample by reflux boiling (16 h) with HCl 6N. Chlorhydric acid is removed by vacuum. The residue is taken up by distilled water and HP is oxidized with chloramine-T. The oxidation product reacts then with 4-dimethyl-amino-benzaldehyde. The red colour is then evaluated by spectrophotometric measurement at 560 nm (ISO 140).

3. STEGEMANN H. and STALDER K. (10d, 17)

Hydrolysis of the test sample by reflux boiling (16 h) with HCl 6N. After neutralization and following oxidation of HP with Chloramine-T and reaction with 4-dimethyl-amino-benzaldehyde, the resulting red colour is measured spectrophotometrically at 558 ± 2 nm (ISO 163)

4. MODIFIED STEGEMANN-STALDER METHOD (10c)

This method is partly based on the Stegemann-Stalder method modified by Hurych-Ghvapil and Möhler-Volley. The test sample is hydrolyzed with H₂SO₄ 6N added with Stanneous Chloride at 110°C for 16 h. (The samples are kept in hydrolysis tubes inside an exsiccator in the oven). After filtration and oxidation of HP as above, the excess of chloramine-T is removed by Perchloric acid. The colour assessment is carried out also with 4-dimethyl-amino-benzaldehyde (ISO 153).

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Previous statistical computation of the results from collaborative test showed that the Stegemann-Stalder method (ISO 163) (10d, 11, 12, 13, 14) is to be preferred as a basis for an ISO-Recommendation thanks to its practical feasibility and good reproducibility.

At the Paris' meeting of the Working Group 2 in March 1971, this view-point has been accepted by the members of the Working Group, but it was requested to proceed with the study of this method, particularly with regard to the influence of hydrolysis period and to the reproducibility.

Since in our laboratory we are daily confronted with the determination of hydroxyproline, and also since the 4 proposed ISO-methods exist now as draft directives from the ECM, we decided to undertake this study immediately. Our aim is not to add a new method to the many ones existing already, but confront it with daily practice.

The colour-reagent has been modified according to ARNETH and HAMM (3). Iso-propanol is more suitable than n-propanol, because the colour stability is better and the blank value is lowered.

To perform our study on hydrolysis time, we included this modification.

The essays described hereunder have been carried out on two products differing widely in composition to allow a better judgement of the validity of the proposed methods.

II. METHODS.

We used the ISO method ISO/TC 34/SC 6/WG 2 - N163 (10d) based on the Stegemann-Stalder method, introducing the modification mentioned in the introduction.

We also used the method published in "Die Fleischwirtschaft" 50, 837 (1970) (8) without including the colour reagent they proposed, which is also based on the Stegemann-Stalder method (Alternative method).

The principal differences between the two methods used are summarized in Table I.

	<u>ISO TC 34/SC 6/WG 2 - N163</u>	<u>Alternative method.</u>
Hydrolysis period	16 hours	7 hours
Reagents	sample + 100 ml HCl 6N	sample + 30 ml of the following solution : 35 g SnCl ₂ + 280 ml H ₂ O + 700 ml HCl (d = 1.19)
Filtration	Diatomaceous earth filter paper S. & S. Nr 287	No specifications. We use S. & S. Nr 589 ²
Neutralization	Neutralization after hydrolysis with NaOH	No neutralization.

III. RESULTS AND DISCUSSION.

1. Sample preparation.

We prepared a sample of Dry Sausage (Salami-type) and another of Corned Beef. They are homogenized by passing twice through a meat mincer with holes of 3 mm, and mixing afterwards with a fork.

Five portions of every product were transferred to hermetically sealed cans and kept in a refrigerator at + 4°C.

This procedure allowed to work, throughout the whole study, with duplicates from an identical sample.

The average compositions of these products are :

TABLE II - Average composition of the products.

	<u>Dry Sausage</u>	<u>Corned Beef</u>
Moisture (%)	30.9	62.2
Proteins on sample as such (%)	19.1	21.8
Fat on sample as such (%)	41.6	12.7

The two products were chosen on behalf of their widely differing fat content to check whether the hydrolysis period is sufficient when the fat content is very high.

2. Procedure.

For every meat sample and for every hydrolysis period, we made three batches. Each batch represents six portions of the same sample, which are hydrolyzed simultaneously.

From each of these hydrolyzed portions we prepared two dilutions ; this leads to 12 analytical results per batch.

Altogether we obtained 108 results for the Dry Sausage and also 108 results for the Corned Beef, subdivided as follows :

<u>Dry Sausage</u> :	ISO 163	16 h of hydrolysis	: 36 results
	ISO 163	7 h of hydrolysis	: 36 results
	Alternative method	7 h of hydrolysis	: 36 results
<u>Corned Beef</u> :	ISO 163	16 h of hydrolysis	: 36 results
	ISO 163	7 h of hydrolysis	: 36 results
	Alternative method	7 h of hydrolysis	: 36 results

All the analyses were performed by the same analyst.

3. Results.

The results are shown in the tables III, IV, V and VI.

TABLE III : Hydroxyproline content in % (ISO 163)

Dry Sausage

Hydrolysis for 16 hours.

<u>1st batch</u>		<u>2nd batch</u>		<u>3th batch</u>	
<u>dil. I</u>	<u>dil. II</u>	<u>dil. I</u>	<u>dil. II</u>	<u>dil. I</u>	<u>dil. II</u>
0,51	0,52	0,52	0,52	0,48	0,45
0,52	0,53	0,49	0,52	0,46	0,48
0,51	0,51	0,52	0,53	0,47	0,47
0,52	0,52	0,51	0,50	0,46	0,48
0,52	0,51	0,49	0,52	0,47	0,49
0,52	0,53	0,53	0,53	0,45	0,49

<u>ISO 163 - 16 h</u>	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
1st batch	0,518	0,51	0,53
2nd batch	0,515	0,49	0,53
3th batch	0,472	0,45	0,49

For the other assays we give only the mean, minimum and maximum values.

Hydrolysis for 7 hours.

<u>ISO 163 - 7 h</u>	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
1st batch	0,513	0,47	0,56
2nd batch	0,552	0,50	0,61
3th batch	0,486	0,47	0,51

TABLE IV : Hydroxyproline content in % - Alternative method - 7 hours.

	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
<u>Dry Sausage</u>			
1st batch	0,613	0,59	0,65
2nd batch	0,609	0,59	0,63
3th batch	0,607	0,59	0,62

TABLE V : Hydroxyproline content in % - (ISO 163)

Corned Beef

Hydrolysis for 16 hours

	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
1st batch	0,646	0,60	0,68
2nd batch	0,605	0,59	0,63
3th batch	0,674	0,66	0,71

Hydrolysis for 7 hours

	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
1st batch	0,838	0,74	1,06
2nd batch	0,693	0,64	0,73
3th batch	0,715	0,67	0,77

TABLE VI : Hydroxyproline content in % - Alternative method - 7 hours.

	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
<u>Corned Beef</u>			
1st batch	0,824	0,79	0,85
2nd batch	0,791	0,75	0,82
3th batch	0,788	0,77	0,83

The results have been computed by variance analysis. This was done on the mean values of the batches since the previous studies showed that the reproducibility within one batch was good, meaning that the factors sampling and dilution were non-significant (11,13).

Variance analysis of our results showed that the factor batch is always non-significant. This means that under the described working conditions the probability of occurrence of a difference of the factor batch due only to chance is below 0.8. Similarly it is possible to evaluate the influence of the hydrolysis period on the results.

Dry Sausage : the factor hydrolysis period is non-significant.

Corned Beef : the factor hydrolysis period is significant on the level of $P = 0,844$

7 h hydrolysis gives higher results than 16 h hydrolysis.

This rather surprising result has been re-ascertained by 2 new batches of 12 determinations each.

TABLE VII : Complementary results of HP determination in Corned Beef with ISO 163

	<u>Mean Values</u>	<u>Minimum</u>	<u>Maximum</u>
16 h	0,65	0,60	0,68
7 h	0,718	0,68	0,75

Comparison of the results from the "Alternative method" with those from ISO method 163 shows that in all cases the results are significantly different, except for the 7 h hydrolysis period with Corned Beef.

IV. CONCLUSIONS.

1. The Alternative method gives higher results than ISO 163 - 16 hours.
Except for Corned Beef, this Alternative method gives also higher results than ISO 163 - 7 hours. However, these latter results are abnormally high as compared to the 16 hours hydrolysis period.
2. It seems that for ISO method 163 the irregularities of the results could be due up to a certain extent to the hydrolysis period.
We intend to proceed with the study of this hydrolysis period for this method with other meat products, since the nature of the product itself seems to influence also the results.
We can not conclude if the Alternative method is better than the ISO 163 method. In a previous study (13) the recovery yields of added HP varies from one laboratory to another between 82,3 and 120,1 %.
For that reason we intend to undertake a similar study with the Alternative method, by adding known quantities of HP to various samples.
Until these points are clarified we propose to maintain a hydrolysis period of 16 hours.

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