

# Improved Method for Determining Food Protein Degree of Hydrolysis

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**ABSTRACT:** When producing hydrolyzed proteins, it is important to determine the degree of hydrolysis (DH). The trinitro-benzene-sulfonic acid (TNBS) method is well established with regard to enzymatic hydrolysis. However, this method is laborious, cannot be used to follow a hydrolysis reaction continuously, and includes hazardous and unstable chemicals. This paper describes a method based on the reaction of primary amino groups with o-phthaldialdehyde (OPA). The conclusion is that the OPA method of analyzing the DH of protein hydrolyses is more accurate, is easier and faster to carry out, has a broader application range, and is environmentally safer than the TNBS method.

**Key Words:** degree of hydrolysis, food protein, hydrolysis, proteolytic, analysis

## Introduction

IN PROTEIN HYDROLYSIS, THE KEY PARAMETER FOR MONITORING the reaction is the degree of hydrolysis (DH). DH is defined as the percentage of cleaved peptide bonds:

$$DH = h/h_{\text{tot}} * 100\%$$

where  $h_{\text{tot}}$  is the total number of peptide bonds per protein equivalent, and  $h$  is the number of hydrolyzed bonds.  $h_{\text{tot}}$  is dependent on the amino-acid composition of the raw material.

Several methods of monitoring the DH during protein hydrolysis have been described in the literature, for example, pH-stat, osmometry, soluble nitrogen content, and the trinitro-benzene-sulfonic acid (TNBS) method. The pH-stat technique (Jacobsen and others 1957) monitors the DH by adding a base (or acid depending on the pH of hydrolysis) to keep the pH constant during hydrolysis. The amount of base used is proportional to the DH. In practical protein hydrolysis experiments, the pH-stat technique is limited to pH conditions higher than around 7 (Adler-Nissen 1986). When hydrolyzing to obtain a high DH above 30%, it is not economically feasible to carry out hydrolysis when using pH-stat (constant pH > 7) as a single enzyme system working efficiently at pH > 7 is not readily available. To obtain a very high DH, a combination of different enzymes is needed. This will include enzymes with highest activity at pH lower than 7, which is out of the range of pH-stat control. Furthermore, the addition of a base during hydrolysis may be undesirable depending on the use of the end product.

During a hydrolysis reaction, the alteration of the mixture's freezing point depression can be measured by an osmometer (cryoscope). This can be correlated to DH (Adler-Nissen 1984). Osmometry is a fast method that can be used for many reactions. Its limitations are that it cannot be used in highly viscous solutions or solutions with a high concentration of solutes, such as salt, used as preservatives during long reactions. The content of nonprotein compounds in the substrate, which are hydrolyzed by other activities (for example, amylase) in the protease preparation, can also make it impossible to correlate osmometer readings with DH values of the protein.

The monitoring of hydrolysis by analyzing the amount of soluble nitrogen in aqueous trichloroacetic acid (SN-TCA) was discussed by Margot and others (1994). They reported a good correlation between SN-TCA and base consumption in a pH-stat where whey protein was hydrolyzed with trypsin. The prerequisite for a good performance of the SN-TCA method seems to be the use of endo-peptidase activity. If the enzyme system includes a major part as exo-peptidase activity, the result will most likely be a less correlation between SN-TCA and the degree of hydrolysis measured by the base consumption. This theory is based on the fact that the action of an exo-peptidase will not result in the same increase in solubility as when the same number of peptide bonds are cleaved by an endo-peptidase. The degree of hydrolysis measured as percentage of peptide bonds cleaved was not stated in the reference.

The TNBS method is based on the reaction of primary amino groups with trinitro-benzene-sulfonic acid (TNBS) reagent (Adler-Nissen 1979). However, the method does have its drawbacks. It is laborious, and it is not possible to obtain results quickly enough during hydrolysis to follow the process closely. In addition, the TNBS reagent is unstable, toxic, and has to be handled carefully due to the risk of explosion. So there is a need for an alternative method without these drawbacks.

To provide a basis for developing a suitable method, a reaction was selected between amino groups and o-phthaldialdehyde (OPA) in the presence of beta-mercaptoethanol forming a colored compound detectable at 340 nm in a spectrophotometer (Figure 1). This OPA method was described in detail by Church and others (1983), who suggested it for following the proteolysis of milk proteins in dairy science research. In our work with the OPA method, the environmentally more acceptable dithiothreitol (DTT) was used instead of  $\beta$ -mercaptoethanol.

Another alteration in the method is the selection of serine as the standard, since in reactions serine shows a response very close to the average response of amino acids. Data for determining the absorbance of the OPA reacted with amino acids and peptides at 340 nm are given in Table 1. It was decided to improve the accuracy of the mean value by omitting figures with more than 15% difference from the overall

**Table 1—Absorption at 340 nm of OPA reacted with amino acids and peptides**

	OD/mmol/100mL	% of mean
Glycine	5835*	82
Alanine	7156	101
Leucine	7310	103
Phenylalanine	7107	100
Serine	7075	100
Cysteine	2311*	33
Methionine	7067	100
Tryptophan	6776	96
Tyrosine	7147	101
Aspartic acid	7297	103
Asparagine	7808	110
Glutamine	7646	108
Lysine	5814*	82
Arginine	7451	105
Histidine	6732	95
N-Glycyl glycine	6869	97
N-Glycyl-glycyl glycine	6099	86
N-Leucyl-methionine	7240	102
N-Lysyl-phenylalanine	7280	103
N-Glycyl-leucyl-tyrosine	6432	91
Mean value	7088	
s	419	

\* Not included in the calculation of mean value

mean value. This included the values for cysteine, glycine, and lysine. Cysteine has previously been mentioned as non-reactive with the OPA-reagent (Church and others 1983). They also reported the absorbance for glycine relatively lower but not as much as we found. OPA reaction with  $\epsilon$ -amino groups of lysine is reported to have the absorption as the  $\epsilon$ -amino group. In any case, we found the absorption value for lysine relatively low compared to the other amino acids tested, except for cysteine.

In this work, the correlation of the OPA method with the widely accepted TNBS method has been investigated, especially in relation to protein hydrolyses with a degree of hydrolysis of up to 65% and using soy protein and Na-caseinate as substrates.

To calculate DH from spectrophotometer readings of the serine standard and the test sample, a formula was developed similar to the formula for the TNBS method reported by Adler-Nissen (1986).

To calculate DH, it is necessary to have values for  $h$  and  $h_{\text{tot}}$  (see above). The expression for  $h$  in the OPA method is:

$$h = (\text{serine-NH}_2 - \beta) / \alpha \text{ meqv/g protein}$$

The values reported by Adler-Nissen (1979) are used here for  $\alpha$  and  $\beta$ .

For most proteins, the average molecular weight of the amino acids is about 125 g/mole, making  $h_{\text{tot}}$  about 8 g equivalents per kg protein. More exact values for  $\alpha$ ,  $\beta$ , and  $h_{\text{tot}}$  given by Adler-Nissen (1986) are quoted later.

Results from analysis with the OPA method were compared to the TNBS method using protein hydrolysates with a DH of up to 65% in order to determine whether the OPA method can be used to monitor DH in protein hydrolysis reactions. The relevance of following the degree of hydrolysis to a very high DH recently has become of interest in the production of flavors. This type of product requires a large content of free amino acids and small peptides, corresponding to a high DH. Traditionally, hydrolyzed vegetable protein (HVP) produced by acid hydrolysis has been used as a flavor

and flavor enhancer. Due to market demands for foods produced with the use of less chemical methods, the option of enzyme hydrolysis has become of interest.

## Materials and Methods

THE TNBS METHOD WAS CARRIED OUT AS DESCRIBED BY Adler-Nissen (1979). The OPA method was conducted as follows:

### Apparatus

Erlenmeyer flasks: 100 mL, 200 mL, and 500 mL. Test tubes: 10 mL. A 4-decimal analytical balance. Pipettes: 400  $\mu$ L, 3 mL, and 4 mL. Magnetic stirrer (Janke & Kunkel, Staufen, Germany). Whirlmixer (Scientific Industries, Bohemia, N.Y., U.S.A.). Spectrophotometer PU8620 (Philips, Cambridge, England) at 340 nm.

### Reagents

The OPA reagent was prepared as follows: 7.620 g di-Na-tetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) were dissolved in 150 mL deionized water. The reagents were completely dissolved before continuing. 160 mg o-phthaldialdehyde 97% (OPA) was dissolved in 4 mL ethanol. The OPA solution was then transferred quantitatively to the above-mentioned solution by rinsing with deionized water. 176 mg dithiothreitol 99% (DTT) was added to the solution by rinsing with deionized water. The solution was made up to 200 mL with deionized water.

The serine standard was prepared as follows: 50 mg serine (Art.7769 Merck, Darmstadt, Germany) was diluted in 500 mL deionized water (0.9516 meqv/L).

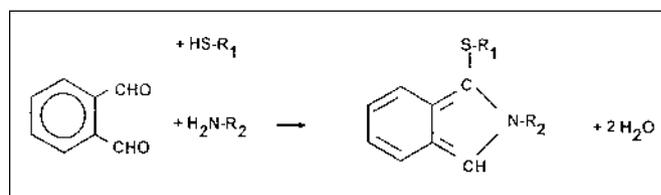
The sample solution was prepared as follows: X g sample was dissolved in 100 mL deionized water. X is 0.1 to 1.0 g sample containing 8% to 80% protein. The DH of the sample also influences the amount required.

### Procedure

All spectrophotometer readings were performed at 340 nm using deionized water as the control.

Three mL OPA reagents were added to all test tubes. Test tubes used for analyzing 1 sample (double determinations) were: standard, 4 tubes; blank, 4 tubes; sample, 4 tubes. As absorbance changes somewhat with time, it is important the samples stand for exactly the same time (2 min) before measuring. The assay was carried out at room temperature.

**Standard measuring.** 400  $\mu$ L serine standard was added to a test tube (time 0) containing 3 mL OPA reagents and mixed for 5 s. The mixture stood for exactly 2 min before being read at 340 nm in the spectrophotometer. Two standards were measured before the blanks along with sample values. The last 2 standards were measured after having determined all blanks and sample values. The mean of these standards



**Figure 1—The OPA-reaction. OPA reacts with primary amino groups and a SH-compound (dithiothreitol, DTT) to form a compound that will absorb light at 340 nm.**

**Table 2—Value of constants  $\alpha$ ,  $\beta$ , and  $h_{\text{tot}}$  for different protein raw materials (Adler-Nissen 1986)**

Protein	$\alpha$	$\beta$	$h_{\text{tot}}$
Soy	0.970	0.342	7.8
Gluten*	1.00	0.40	8.3
Casein	1.039	0.383	8.2
Whey*	1.00	0.40	8.8
Gelatin	0.796	0.457	11.1
Meat*	1.00	0.40	7.6
Fish*	1.00	0.40	8.6

\* When raw material has not been examined, then  $\alpha$  and  $\beta$  are estimated to be 1.00 and 0.40, respectively.

was used for calculations. The typical value of the standards was OD about 0.8.

**Blank measuring:** Blanks were prepared from 400  $\mu\text{L}$  deionized water and treated as described above. The typical value of a blank was OD about 0.07.

**Sample measuring:** Samples were prepared from 400  $\mu\text{L}$  sample.

### Calculation

#### Determination of h:

$$\text{Serine-NH}_2 = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \cdot 0.9516 \text{ meqv/L} \cdot 0.1 \cdot 100 / X \cdot P$$

where serine-NH<sub>2</sub> = meqv serine NH<sub>2</sub>/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in liter (L). h is then:  $h = (\text{serine-NH}_2 - \beta) / \alpha$  meqv/g protein, where  $\alpha$  and  $\beta$  are shown in Table 2 for specific raw materials.

#### Calculation of DH:

$$\text{DH} = h / h_{\text{tot}} \cdot 100 \%$$

where  $h_{\text{tot}}$  for specific raw materials is found in Table 2.

### Preparation of Hydrolyses

To compare the TNBS method with the OPA method, hydrolyses of Na-caseinate and soy protein isolate were prepared. The raw materials used were Na-caseinate (Miprodan 30; MD Foods, Viby, Denmark) and soy protein isolate (PP500E; Protein Technologies International, St. Louis, Mo., U.S.A.). The raw materials were suspended in deionized water with a protein concentration of 8%. The solutions were pasteurized at 85 °C for 3 min and cooled to the hydrolysis temperature (50 °C). The pH was adjusted to 8.0 using 4 N NaOH before adding the enzymes, and no later adjustments of pH was made. The enzymes used were Alcalase® 2.4 L, Neutrase® 0.5 L, and Flavourzyme® 3870LAPU/g (Novo Nordisk A/S, Bagsvaerd, Denmark), dosages of 1% and 2% of protein content, respectively. During hydrolysis pH drops from 8 to about 6 (data not recorded). Following enzyme addition, samples were taken after 1.5, 2, 3, and 20.5 h, heated to 85 °C for 3 min to inactivate the enzymes, and frozen for later analysis.

### Results and Discussion

TO DETERMINE THE STANDARD DEVIATION (S) OF THE OPA method and compare it with the S of the TNBS method, 10 individual analyses of all samples were performed using both methods. The unhydrolyzed sample was

**Table 3—DH determinations of soy protein hydrolysate and Na caseinate hydrolyzate measured by the OPA method and the TNBS method**

Time of hydrolysis	Soy protein isolate		Na-caseinate		
	OPA DH, %	TNBS DH, %	OPA DH, %	TNBS DH, %	
0 h		-1.35		-0.30	
		-0.97		-0.21	
		-0.89	2.22	-0.21	
		-0.94	2.20	-0.18	
		-0.79	2.47	-0.12	
		-1.06	2.55	-0.07	
		X = -1.53	X = -1.00	X = 2.36	X = -0.18
		s = 0.291	s = 0.193	s = 0.176	s = 0.080
	1.5 h	30.32	29.78	34.45	34.56
		30.11	30.18	34.49	34.33
30.11		30.83	34.30	34.04	
30.08		30.18	34.49	34.10	
29.90		30.18	34.38	34.04	
30.90		30.04	34.30	34.74	
29.94		29.58	34.38	34.16	
29.80		29.98	34.61	34.39	
30.08		30.31	34.26	34.27	
29.97		30.04	34.07	34.50	
	X = 30.12	X = 30.11	X = 34.37	X = 34.31	
	s = 0.309	s = 0.332	s = 0.151	s = 0.237	
2 h	32.96	33.70	38.13	37.72	
	32.96	33.70	37.60	37.14	
	33.07	32.71	37.56	37.85	
	33.11	34.15	37.73	37.78	
	32.92	33.01	37.65	37.46	
	32.96	33.24	37.51	37.72	
	33.23	34.15	37.20	37.65	
	32.96	32.79	37.60	37.98	
	33.00	33.32	37.69	37.14	
	32.96	34.07	38.26	38.10	
	X = 33.01	X = 33.48	X = 37.69	X = 37.65	
	s = 0.096	s = 0.549	s = 0.303	s = 0.322	
3 h	38.84	38.83	42.98	43.14	
	38.71	38.14	42.74	42.99	
	38.57	39.61	42.74	42.61	
	38.57	38.31	42.74	43.29	
	38.44	38.40	43.03	42.17	
	38.39	39.00	42.70	42.84	
	38.62	39.00	42.79	43.51	
	38.53	38.05	42.84	43.36	
	38.39	39.18	43.27	42.47	
	38.57	39.18	42.74	44.03	
	X = 38.56	X = 38.77	X = 42.86	X = 43.04	
	s = 0.141	s = 0.518	s = 0.183	s = 0.546	
20.5 h	60.75	62.51	60.33	62.30	
	60.82	60.85	60.24	61.53	
	61.47	63.06	60.18	63.45	
	61.47	63.19	59.35	62.81	
	61.54	61.09	60.12	60.89	
	61.47	62.64	60.83	62.43	
	61.47	65.40	60.53	65.50	
	60.90	64.02	60.71	63.19	
	60.90	60.30	60.06	61.66	
	61.54	64.85	60.59	62.43	
	X = 61.23	X = 62.79	X = 60.33	X = 62.62	
	S = 0.340	s = 1.692	s = 0.442	s = 1.275	

analyzed by 5 individual analyses. The results of individual analyses, the average, and S are given in Table 3.

In Table 3, the values for measuring the DH in raw materials and hydrolysates up to a DH of 63% are shown. The DH of the unhydrolyzed material was slightly different from 0. A deviation from 0 can be explained 2 ways.

Both the OPA and the TNBS are reacting with  $\epsilon$ -amino

groups. When DH is 0, there are options of reactions with the  $\epsilon$ -amino groups (lysine) and, to a minor extent, the amino groups at the end position of the protein molecule.

Background color from the samples might interfere with the color formed by the reagent.

The data suggest that the OPA method is more reactive toward the  $\epsilon$ -amino groups in casein than in soy. The difference in the availability of the  $\epsilon$ -amino groups might explain this, as the content of lysine in casein and soy is similar (8.1% and 6.2%, respectively, of the total protein; Adler-Nissen 1986). The availability of the  $\epsilon$ -amino groups can be reduced significantly by Maillard reactions in the proteins, for example. Both the OPA and TNBS methods should be used with caution at very low DH.

The relative  $s$  is defined as  $s$  divided by DH and is shown in Figure 2 (soy protein hydrolysate) and Figure 3 (Na-caseinate hydrolysate) to compare the accuracy of the 2 methods.

For both raw materials, the relative  $s$  is noticeably less when using the OPA method of analysis than the TNBS meth-

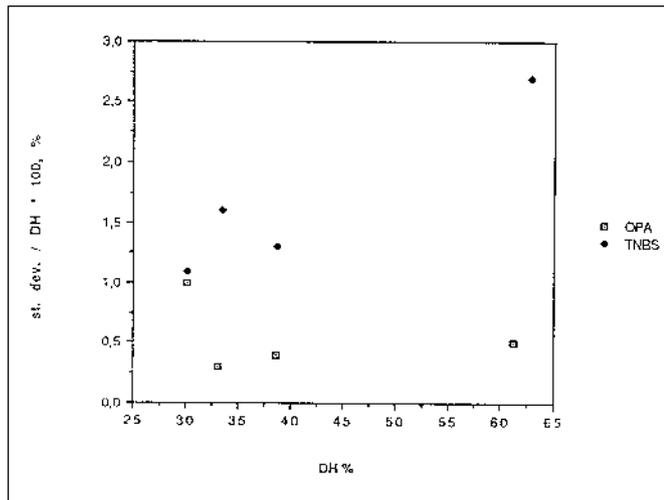
od. For the OPA method, the relative  $s$  is less than 1%. The TNBS method has a relative  $s$  of up to 2.5%, with a tendency to a higher  $s$  for a higher DH. This shows the OPA method is more reliable at a high DH than the TNBS method.

Correlation plots of the average DH found by TNBS against OPA method for the 2 raw materials are given in Figure 4 and Figure 5, respectively. The correlation between the OPA and TNBS methods for the 2 raw materials is very good ( $r > 0.999$ ). The correlation between the 2 methods is also slightly different for each raw material. A similar raw material dependency was reported by Adler-Nissen (1979), who investigated the accuracy of the TNBS method. As DH increases, more and more free amino acids will be formed. The types of amino acids released will be different from 1 protein to another depending on the protein composition of the raw material. From our data in Table 1, the absorption at 340 nm of colored compounds from reaction with glycine, cysteine, and lysine is relatively low. The different content of these 3 amino acids in soy and casein is expected to be reflected in the content of free amino acids in the hydrolysates. From our experience, the glycine and cysteine both are staying bounded in peptides to a larger extent than most of the other amino acids when the proteins are hydrolyzed to a high DH. Thus, we expect the differences found to be largely explained by the lysine content.

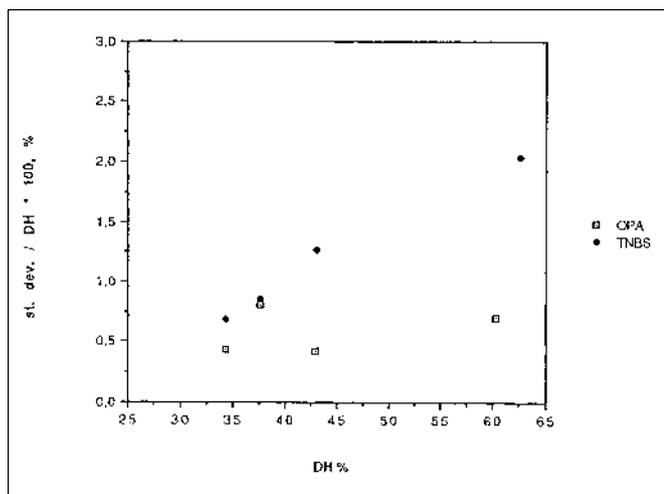
For practical applications, it should be noted that some of the food proteins have compositions that can cause problems in determining the DH. Wheat gluten has a large content of cysteine and low content of lysine, and gelatine has a large content of glycine. Adler-Nissen (1979) showed this difficulty clearly for gelatine where the slope of the correlation equation between TNBS and hydrolysis in a pH-stat was very different from soy and casein.

The constant term in the correlation equations also shows a raw material dependency. This is due to the inaccuracy that arises when measuring unhydrolyzed (DH = 0) samples as discussed above.

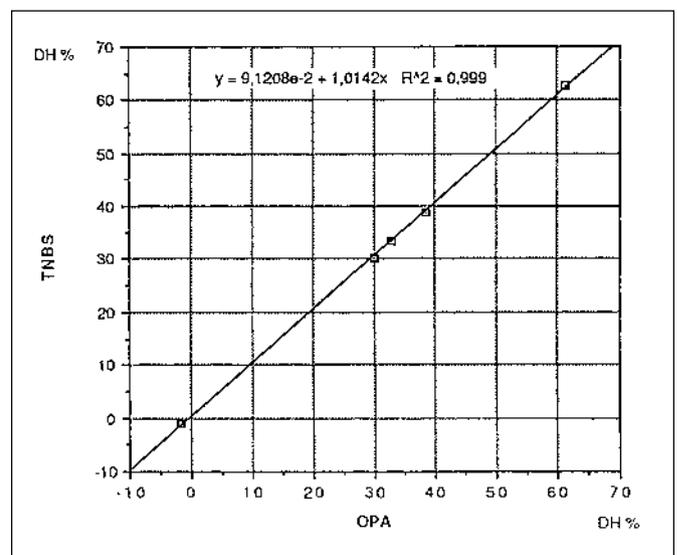
It should be noted that the OPA method has several advantages over the TNBS method. The reagent used to form the colored compound in the reaction with amino groups



**Figure 2—Relative standard deviation for measurements of DH in soy protein hydrolysates**



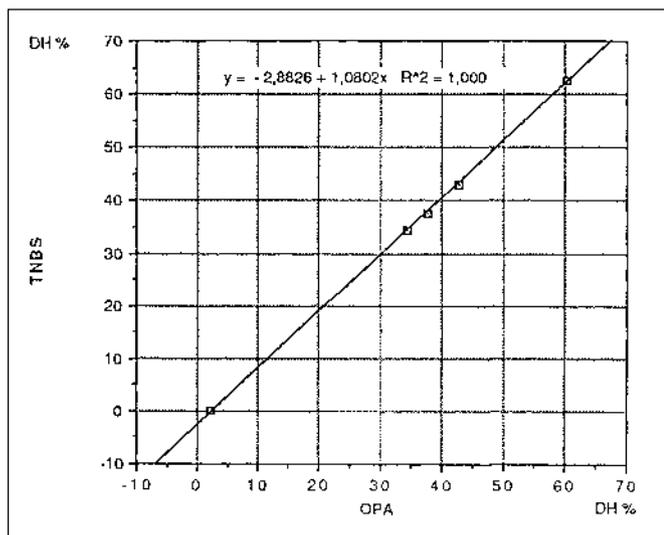
**Figure 3—Relative standard deviation for measurements of DH in Na-caseinate hydrolysates**



**Figure 4—Correlation between the TNBS method and the OPA method for soy protein hydrolysates**

has a very different stability. The TNBS reagent is rather unstable, and the solutions prepared for the analysis have to be kept away from light or they will develop a color that influences measurements. In current practice, the test tubes are covered, for example, by aluminum foil. This procedure is time-consuming. Until recently the TNBS reagent was sold as a solid compound, but due to the risk of explosion, it is now delivered as a solution. Stored in a solution, the TNBS reagent is slightly colored resulting in a higher blank value than previously when the solid reagent was used. (The analysis described in this paper was based on the solid TNBS reagent.)

Another important difference between the 2 methods is the time at which a sample is taken until results can be obtained. In the OPA method, the reaction time before measuring absorbance is 2 min, whereas the TNBS reaction takes 1 h. This means only the OPA method can be used to monitor DH continuously during the reaction and then as a production control.



**Figure 5—Correlation between the TNBS method and the OPA method for Na-caseinate hydrolyzates**

Examples of practical applications of the method are protein extracts produced for applications as functional ingredients and/or as flavor. Production of flavor components from protein raw materials like soy, yeast, and meat require a DH of 30% or more. A very high DH is found in soy sauce (about 70%) and in yeast extracts (above 50%). The OPA method has been successfully applied in these types of products. Also, protein extracts with a DH of 15% to 20% can be analyzed with the OPA method. Extracts of meat and fish raw materials are produced today in the industry to be applied as ingredients in processed fish and meat products. A last example is production of protein hydrolyzate as an ingredient in dietetic foods, baby-food formulas, and sports drinks. These types of products have a wide range of DH from about 10% to 50%.

### Conclusions

THE NEW METHOD HAS PROVED TO BE MORE ACCURATE THAN the previous method based on TNBS reaction. Furthermore, the OPA method can be used to follow the hydrolysis reaction during hydrolysis and is much less time-consuming than the TNBS method. Because results are available 2 min after the sample is taken, the OPA method can be used in production to monitor DH during hydrolysis. A final benefit is that the OPA reagent is more stable and less toxic than the TNBS reagent. It is therefore suggested that the OPA method is used for determining DH in hydrolyzed proteins, in food products, as well as feed products.

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