

Date issued:
June 2002

Title:

MILK POWDERS & SIMILAR PRODUCTS

LI number
08.088-1

Replaces:
LI-08.088

References:
NRC/QS/lGr/lbe

DEGREE OF HYDROLYSIS TNBS METHOD

Page: 1/11

Quality criteria: hydrolysis, degree of

1 SCOPE AND FIELD OF APPLICATION

Determination of the degree of hydrolysis in protein hydrolysates and protein hydrolysate based infant formulas by determination of the amino nitrogen by reaction with trinitrobenzenesulfonic acid. The method is based on the procedure described by Adler-Nissen (reference section 14.1).

2 DEFINITIONS AND ABBREVIATIONS

2.1 Definitions

The degree of hydrolysis is defined as the percentage of amino nitrogen on total nitrogen.

2.2 Abbreviations

TNBS: Trinitrobenzenesulfonic acid

SDS: Sodium dodecylsulfate

TN: Total nitrogen

Leu: Leucine

N-NH₂: Amino nitrogen

DH: Degree of hydrolysis

3 PRINCIPLE OF THE METHOD

Dissolution of the sample in 1 % SDS solution and appropriate dilutions. Reaction of primary amino groups with TNBS solution. Measurement of absorbance of the reaction products at 340 nm.

Comparison with a calibration curve established with leucine. Calculation of the degree of hydrolysis.

4 SAFETY PRECAUTIONS

See enclosure 1

5 CHEMICALS AND MATERIALS

Commercial references are only a guideline. Numbers in the margin refer either to the Merck's chemicals and reagents catalogue or to that of Nestlé laboratory material.

5.1 Chemicals

Before using chemicals refer to the Sigma/Aldrich Guide to Chemical Safety and/or other adequate manuals or safety data sheets approved by your local authorities. Read carefully the safety instructions given in Enclosure 1. More detailed safety information can also be obtained from NRC/QS.

- 109060 - Hydrochloric acid 0,1 M in solution (CAS no 7647-01-0)
- 106580 - di-Sodium hydrogen phosphate dihydrate (CAS 10028-24-7)
- 106346 - Sodium dihydrogen phosphate monohydrate (CAS no 10049-21-5)
- 105360 - L-leucine (CAS no 61-90-5)
- Dodecylsulfate-Na salt (SDS), Serva 20760 (CAS no 151-21-3)
- 2,4,6 - Trinitrobenzenesulfonic acid (TNBS) 1M, Fluka 92823 (CAS no 2508-19-2)

5.2 Materials

Standard laboratory glassware

- 334007 - Volumetric flask amber 50 ml, wide neck, with RN
- 126303 - Test tube amber with stopper, 15 ml
- 101704 - Centrifuge tube, 100 ml
- 622707 - Filter unit disposable, MILLEX-HV, 0,45 µm, Ø 25 mm, low protein binding Durapore, SLHV 025LS
- 333001 - Cuvettes, single use, 10x10x45mm, 4 ml volume
- 911901 - Vessel, stainless steel, vol. 7-12 l, Haake W13
- 914103 - Thermostat, Haake DC30
- 900405 - Test tube mixer Vortex
- 910702 - pH-meter Metrohm E691
- 823207 - Electrode for pH, pt 1000 Metrohm 6.0204.000
- 904201 - Centrifuge with rotor "Heraeus" Labofuge 400
- 904202 - Adapter for 100 ml tubes
- 912802 - Spectrophotometer LKB Ultrospec

6 PREPARATION AND CHECK OF REAGENTS
--

6.1 SDS solution, 1 % (w/v)

Into a 1 000 ml volumetric flask weigh 10,0 g SDS (dodecylsulfate Na salt). Dissolve and make up to the mark with distilled water.

6.2 Sodium phosphate buffer 0,2M, pH 8,2

6.2.1 Solution 1

Into a 1 000 ml volumetric flask weigh 35,59 g of di-sodium hydrogen phosphate dihydrate. Dissolve and make up to the mark with water. This solution keeps one week at 4 °C.

6.2.2 Solution 2

Into a 500 ml volumetric flask weigh 13,80 g of sodium dihydrogen phosphate monohydrate. Dissolve and make up to the mark with water. This solution keeps one week at 4 °C.

6.2.3 Sodium phosphate buffer: Working solution

Pour 500 ml of solution 1 (6.2.1) into a 1 000 ml beaker and adjust to pH 8,2 with solution 2 (6.2.2). Prepare this solution freshly each day.

6.3 TNBS solution, 0,1 % (w/v)

Pipette into a 50 ml amber volumetric flask 170 µl of TNBS 1M (trinitrobenzenesulfonic acid). Dilute and make up to the mark with distilled water. Prepare a fresh solution every day and keep the solution in the dark.

6.4 Leucine standard solutions

6.4.1 Leucine standard solution, 10 mM

Into a 50 ml volumetric flask weigh exactly 65,6 mg L-Leucine. Dissolve and adjust to the mark with SDS 1 % (6.1). This solution keeps for one week at 4 °C.

6.4.2 Leucine standard solution, 1mM

Pipette into a 50 ml volumetric flask 5 ml of the leucine standard solution, 10 mM (6.4.1). Adjust to the mark with SDS, 1 % (6.1). This solution keeps for one week at 4 °C.

6.4.3 Leucine standard solutions for calibration curve

To obtain the required concentrations pipette the following volumes of standard solutions 6.4.1 and 6.4.2 into 10 ml volumetric flasks and make up to the mark with SDS, 1 % (6.1).

Leucine standard solution	Volume pipetted	Leucine in nmol / 250 μ l
6.4.1	3 ml	750
6.4.1	2 ml	500
6.4.2	use solution 6.4.2 as is (10 ml)	250
6.4.2	5 ml	125
6.4.2	4 ml	100
6.4.2	2 ml	50

7 APPARATUS

Spectrophotometer capable of measuring absorbances at 340 nm.

8 SAMPLING AND SAMPLE PREPARATION**8.1 Sampling procedure**

Take a representative amount of product.

8.2 Sample preparation

Mix the laboratory sample well before taking out the test portion. Heterogeneous products must be homogenized using a food blender.

9 PROCEDURE

Analysis should be carried out in duplicate.

9.1 Total nitrogen (TN) determination

Determine total nitrogen according to LI-00.556.

9.2 Sample preparation

Into a 100 ml volumetric flask weigh exactly about 0,5 g of powdered product or an amount corresponding to 0,5 g of dry matter of a liquid product. Dissolve and adjust to the mark with SDS 1 % (6.1). Heat the flask for 15 min in a water bath at 50 °C. Shake the flask and let it cool to room temperature. Pour the solution into a centrifuge tube and centrifuge for 20 min at 3 000 g.

9.3 Dilutions

Make 2 dilutions (see table below).

Pipette the following volumes of sample solution (supernatant, 9.2) into 10 ml volumetric flasks. Make up to the mark with SDS, 1 % (6.1).

	dilution 1	dilution factor (d)	dilution 2	dilution factor (d)
Partially hydrolysed infant formula	as is	1	5 ml	2
Extensively hydrolysed infant formula	5 ml	2	3 ml	3.33
Protein hydrolysate	3 ml	3.33	2 ml	5

Filter the solution through a low protein binding filter, 0,45 µm.
Filter one more time if the filtrate is still turbid.

9.4 Reaction and spectrophotometric measurement

Introduce in the respective test tubes by means of a pipette: 250 µl of SDS, 1 % (6.1) for the blank or 250 µl of each calibration standard (6.4.3) or 250 µl sample dilution (9.3).

Add subsequently to each tube 2 ml of sodium phosphate buffer (6.2.3) and 2 ml of TNBS solution, 0,1% (6.3).

Close the tubes and mix them by means of a Vortex. Place the tubes in a water bath at 50 °C for 60 min.

Cover the water bath with aluminium foil.

After 60 min stop the reaction by adding to each tube 4 ml HCl 0,1M.

Mix by means of a Vortex and let the tubes cool to room temperature for 30 min.

Measure the absorbance at 340 nm against air.

9.5 Time of analysis

5 samples in duplicate can be analysed in one working day.

10 CALCULATIONS

10.1 Standard curve

Subtract the absorbance of the blank from the absorbances of the leucine standard solutions.

Plot the obtained absorbances values against the concentration of leucine (in nmol / 250 µl) and draw the calibration line (forced through zero intercept).

Calculate the correlation coefficient (r^2) which should be $> 0,9950$. Otherwise repeat reaction and measurement (9.4).

10.2 Calculation and expression of results

Subtract the absorbance of the blank from the absorbance of the sample and read the leucine concentration on the standard curve (x_1).

The absorbances of dilution 1 and dilution 2 must be within the range of the standard curve. Otherwise repeat with an appropriate dilution.

The amino nitrogen, (N-NH₂), expressed in g / 100 g product, corresponds to:

$$\frac{x_1 \cdot 14,007 \cdot V_1 \cdot d}{m \cdot V_2 \cdot 10000}$$

The hydrolysis degree (DH), i.e. the percentage of amino nitrogen on total nitrogen (%N-NH₂ of TN) corresponds to:

$$\frac{x_1 \cdot 14,007 \cdot V_1 \cdot d \cdot 100}{m \cdot V_2 \cdot TN \cdot 10000}$$

where:

- x_1 = nmol leucine read on the curve (nmol in cuvette).
- m = weight of the test portion, in g.
- V_1 = volume used for sample preparation (9.2) (100 ml).
- V_2 = volume used for the reaction (250 μ l).
- d = dilution factor (9.3).
- 14,007 = atomic weight of nitrogen.
- TN = total nitrogen in g/100g.

Calculate the mean value of the results of degree of hydrolysis (% N-NH₂ of TN) obtained on dilution 1 and dilution 2.

Express results with one decimal.

11 PERFORMANCE CHARACTERISTICS

11.1 Limit of quantification

The limit of quantification corresponds to the absorbance of the first point of the standard curve (50 nMol leucine).

For soya isolates the limit of quantification is estimated to be about 1 % DH.

11.2 Repeatability

The difference between 2 duplicates must not exceed:

0,70 % N-NH₂ of TN for extensively and moderately hydrolysed products

0,20 % N-NH₂ of TN for slightly hydrolysed products

which corresponds to the repeatability limit, *r*, at 95 % confidence level (robust statistics).

These values are given as reference values and should be regularly verified in your own laboratory by your own internal control plan (ICP) for each product type. Refer to LI-00.925 to calculate QC chart limits or use Ester 2, statistic calculation program.

12 ANALYTICAL FLOW SHEET

See Enclosure 2.

13 INTERNAL CONTROL PLAN

Include in each series the analysis of a reference sample with an established reference value according to LI-00.920. Sample can be stored in a dessicator at room temperature for at least 2 years.

14 REFERENCES

14.1 Bibliography

- Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid: J. Agric. Food Chem., 27:1256-1262.
- Internal method R&D-R / FST / DPS January 97: Dosage de l'azote α -aminé.

14.2 Lis mentioned

LI-00.556: Total nitrogen according to Kjeldahl with Büchi system.

LI-00.920: Use of reference samples.

LI-00.925: Calculation and use of analytical precision values, repeatability and reproducibility.

15 MODIFICATIONS FROM PREVIOUS VERSION

Chapter 6.2.2 : solution 2

Chapter 6.4.1 : Leucine standrad solution, 10 mM

Chapter 9.3 Filtration after the dilutions

16 APPROVAL OF THE METHOD

Approved by	Initials	Date
Author	IGR	03.07.02
Group Leader	CH	4.7.02
Head of Department	RAS	1.7.02
CT-QM	RAS	4.7.02

17 ENCLOSURES

- 1 Safety instructions
- 2 Analytical flow sheet
- 3 Analytical flow sheet - Blank form

SAFETY INSTRUCTIONS

(This information refers to occupational exposure)

CAS N°	COMPOUND	HAZARDS	PRECAUTIONS	DISPOSAL
7647-01-0	Hydrochloric acid	Highly toxic. Corrosive.	Use concentrated acid only in a chemical fume hood. Avoid inhalation. Avoid contact.	Neutralise.
10028-24-7	Di-Sodium hydrogen phosphate dihydrate		Avoid contact. Avoid inhalation.	Chemical waste: Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with afterburner and scrubber.
10049-21-5	Sodium dihydrogen phosphate monohydrate	Irritant.	Avoid contact. Avoid inhalation.	Chemical waste: Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with afterburner and scrubber.
61-90-5	L-Leucine		Use in a chemical fume hood. Avoid contact. Avoid inhalation.	Chemical waste: Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with afterburner and scrubber.
151-21-3	Dodecyl sulfate, Sodium salt (Sodium dodecyl sulfate) (Sodium lauryl sulfate)	Irritant. Sensitiser. May cause headache, nausea and vomiting.	Avoid contact. Avoid inhalation.	Chemical waste: Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with afterburner and scrubber.
2508-19-2	2,4,6-Trinitrobenzene sulfonic Acid	Toxic. May Cause Cancer. Irritant.	Use in a chemical fume hood. Avoid contact. Avoid inhalation. Avoid prolonged or repeated Exposure.	Chemical waste: Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with afterburner and scrubber.

Bold: Particularly toxic - Special precautions required

ANALYTICAL FLOW SHEET

Steps

Critical points

Determine total nitrogen (LI-00.556).

Weigh test portion: 0,5 g powder or 0,5 g dry matter.
Dissolve and make up to 100 ml with SDS 1 %.

Perform analysis in duplicate.

Heat in a water bath at 50°C for 15 min.
Cool to room temperature.

Centrifuge at 3000 g for 20 min.

Make appropriate dilutions with SDS 1%.
Filter through low protein binding filters, 0,45 µm.

9.3.1 or 9.3.2 or 9.3.3 or 9.3.4

Reaction:
250 µl SDS 1% (blank)(6.1) or 250 µl calibration standards (6.4.3) or 250 µl sample dilutions (9.3).
2 ml sodium phosphate buffer,
2 ml TNBS 0,1 %.
Mix and leave at 50°C for 1 h.
Stop reaction with 4 ml HCl 0,1M.
Cool to room temperature for 30 min.

Cover the water bath with aluminium foil.

Read the absorbance at 340 nm against air.

Plot the standard curve (10.1)
Calculate the degree of hydrolysis in % of amino nitrogen on TN.

Calculate the mean value of the degree of hydrolysis obtained for dilution on 1 and dilution 2.

ANALYTICAL FLOW SHEET - BLANK FORM

Steps

Critical points
