Sample Preparation

of Proteins and Peptides
by basic Hydrolysation
(for determination of Tryptophan)

DRAFT

Description:

This procedure describes the preparation of protein and peptide containing samples by basic hydrolysation for the qualitative and quantitative determination of tryptophan.
For the analysis of the proteinogenic amino acids it is required to dissociate the amino acid polymer without degradation of the amino acids tryptophan. The proteins and peptides are digested completely by hydrolysing the peptide bond between two amino acids with sodium hydroxide.
For reliable quantitative results it is important that the sample preparation procedure is strictly complied regarding reaction times and temperatures.
During the hydrolysis all other amino acids are decomposed almost completely. Due to this the quantitative determination of other amino acids is not possible. For the determination of the other proteinogenic amino acid use either the oxidative hydrolysis (see SOP: Protein sample preparation by acidic hydrolysis with oxidation, Sykam QM-4160) if the values of the oxygen sensitive amino acids methionine and cystine are required or in case that these amino acids are not of higher interest the simple acidic hydrolysation procedure (see SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159).
If the time period between sample preparation and measurement is longer than a few hours, the prepared sample should be store at 2-8 °C or colder.

Material:

Equipment:

- hydrolysis vessels* or Pyrex glass bottles with screw cap, volume: ≥ 50 ml
- beakers*, volume: 250 ml
- volumetric glass flasks*, volume: 200 ml
- two volumetric glass flasks, volume: 1000 ml
- two plastic bottle for chemicals, volume: 1000 ml
- dash bottle, volume: 1000 ml
- adjustable micro pipets (e.g. Eppendorf pipets), maximum volumes 1000 µl, 200 µl and if internal standard should be used 10 µl
- PTFE coated magnetic stir bar
- PTFE coated stir bar catcher
- oven, block thermostat, microwave oven or similar heating device for hydrolysis vessels
- pH meter incl. pH probe and temperature sensor
- magnetic stirrer
- centrifuge, minimum 14 kpm, volume of centrifuge tubes: 1.5 ml or bigger
- vortex stirrer for micro centrifuge tubes and vials
- refrigerator at 2-8 °C or similar cooling device

Chemicals:

- deionized water or higher grade (conductivity ≤ 0.5 µS)
- sodium hydroxide, analysis grade or higher
- phenol, analysis grade or higher
- amino acids used as internal standard (ISTD) (e.g. D,L-Norleucine, CAS: 616-06-8)

Consumables:

- disposable syringe, volume: 5 ml
- disposable syringe filter, pore size: 0.45 µm
- Sample vials, glass, screw cap with perforated Silicon/PTFE septum (Sykam Cat. No.: S000212)
- centrifuge tubes
- tips for micro pipets, in required sizes
- Sykam Sample Dilution Buffer for physiological samples (Sykam Cat. No.: S000015)
- Amino acid standard mix for hydrolysis
  - Sykam Standard Type H 18 amino acids (calculates in µmol/ml) (Sykam Cat. No.: S000029)
  - Sykam Standard Type H-G 18 amino acids (calculated in µg/ml) (Sykam Cat. No.: S000035)

* one per sample which are prepared simultaneously

Procedure:

Pre-preparation:

1. Basic Hydrolysis Solution 1
   Prepare a solution of 4.2 N sodium hydroxide in water. Therefore weigh in 168 g sodium hydroxide (MW_{NaOH} 40.00 g/mol) in a 1 litre volumetric flask and add about 800 ml deionized water. As soon as the solution reaches room temperature again fill up to 1 litre with deionized water. Transfer this solution in a 1 litre plastic bottle.
2. Acidic Neutralization Solution
   Prepare a solution of 6N hydrochloric acid. Therefore fill 590 ml hydrochloric acid (32 $_{mass}$%, 1.16 kg/l, MW_{HCl} 36.46 g/mol) in a 1 litre volumetric flask and fill up to 1 litre with deionized water.
3. Acidic Neutralization Solution
   Prepare a solution of 1N hydrochloric acid. Therefore fill 89 ml hydrochloric acid (32 $_{mass}$%, 1.16 kg/l, MW_{HCl} 36.46 g/mol) in a 1 litre volumetric flask and fill up to 1 litre with deionized water.
4. Internal Standard Solution
   For preparation of internal standard solutions see SOP: Preparation procedure for Amino Acid Standard Additions QM-4101.

All these solutions except internal standard solutions can be stored for about 2 years. For advised storage time of internal standard solution see related SOP.

Sample pre-preparation:

- Use representative subset of the sample and homogenize it by grinding till the particle size is approximately ≤ 0.5 mm.
- If the sample material has a high moisture concentration,
  - weigh in a few gram or less of sample,
  - dry the sample at a temperature of maximum 50 °C or freeze dry till weight remains constant,
  - determine the weight loss during drying for later calculations if required.
- If the sample material have a high fat content (> 5 %)
  - weigh in a few gram or less of dry sample,
  - reduce the fat concentration by extraction using petroleum ether (e.g. liquid-liquid extraction (LLE) or Soxhlet extraction),
  - remove solvent completely after extraction by evaporation at room temperature,
  - determine the weight loss during defatting for later calculations if required.
- Determine the Kjeldahl-nitrogen content, if possible.
- Otherwise determine the overall amino acid content in a pre-test measurement and estimate the Kjeldahl-nitrogen content from this value. The conversion factor for amino acids to Kjeldahl-nitrogen is 0.16 (this factor may vary depending on the kind of sample material). Also for pre-test measurements follow this SOP.

Sample hydrolysation:

- Weigh in 0.1 to 1.0 g of the sample material, it should be equivalent to approximately 10 mg Kjeldahl-nitrogen.
  - For pre-test sample preparation weigh in 0.1 to 0.3 g depending on the expected protein contend.
- Transfer the sample into a reaction vessel or Pyrex flask.
- Add XX ml of basic hydrolysation solution to the sample
- At this point of sample preparation an internal standard can be added if required (e.g. add 1000 µl of internal standard solution (e.g. norleucine) with a concentration of 4 µmol/ml to the sample). Notice the change in dilution factor.
- Mix the sample carefully with the hydrolysis solution by swivelling.
  - Take care that nearly no sample residues stick to the inner wall of the flask.
- Heat the sample in the closed vessel for 24 h at a temperature of 110 ± 2 °C. See different time and energy settings for microwave hydrolysis in related manual.
  - If not using pressure resistant vessels release overpressure after 30 minutes of heating!
    !!!BE CAREFUL FLASK MAY EXPLODE!!!
- After thermal treatment take the flask out of the oven and cool it down to room temperature by dint of cold water or ice cooled water. If not cooled automatically by microwave

Sample post preparation:

- Transfer the hydrolysate solution quantitatively into a 250 ml beaker with as less as possible SDB/Na (Sample Dilution Buffer), for convenience the SDB can be filled into a dash bottle.
- Use a PTFE stirring bar and a magnetic stirrer to mix the hydrolysis solution during pH adjustment.
- Use a temperature probe (e.g. from pH meter) to observe the temperature of the hydrolysis solution during pH adjustment.
- Add slowly about 18 ml of Neutralisation Solution 1 (preparation see above subsection: Pre-preparation).
  - Thereby the temperature should not exceed 40 °C.
- Use a calibrated pH probe to adjust the pH value of the hydrolysis solution more precisely.
  - Note that the pH value is influenced by the temperature and the temperature-correction for the pH meter may cover only a narrow operating range.
- Continue pH adjustment by adding carefully Neutralisation Solution 1 or 2 to the hydrolysis solution in order to achieve a pH value of 2.1 to 2.3.
- Transfer the pH-adjusted solution with SDB/Na quantitatively into a 200 ml volumetric flask.
  - Thereby rinse the stirring bar and the stir bar catcher as well as the temperature and pH probe with SDB/Na for convenience the SDB can be filled into a dash bottle.

- Fill the graduated flask up to 200 ml with SDB/Na at the respective calibration temperature of the flask.
- *Alternative pH adjustment (simplified but not that precise, retention times may vary)*
  - *Transfer the hydrolysate solution quantitatively into a 200 ml volumetric flask with SDB/Na (Sample Dilution Buffer), for convenience the SDB can be filled into a dash bottle.*
  - *Add slowly about 18.5 ml of Neutralisation Solution 1 (preparation see above subsection: Pre-preparation).*
  - *Thereby the temperature should not exceed 40 °C.*
  - *Fill the graduated flask up to 200 ml with SDB/Na at the respective calibration temperature of the flask.*
  - *Note: Sample pH is not reproducible also after dilution with SDB, this may cause variations of retention times in the acidic range of the separation.*
- If the sample is not measured within a few hours, store the volumetric flask at a temperature of 2 to 8 °C until usage.
- Cleaning of sample solution from particles can be performed by one of the following steps:
  - Take an aliquot of the hydrolysis solution with a syringe and percolate the solution through a 0.45 µm pore size syringe filter.
    - Discard the first percolated millilitre of the solution!
  - Take an aliquot of the hydrolysis solution and transfer to a centrifuge tube and centrifuge solution at 14 kpm for 10 minutes.
- Dilute the cleaned hydrolysis solution with SDB/Na into a sample vial.
  - The dilution factor should be chosen to obtain sufficiently strong signal intensities in the range of the calibration (e.g. 500 µl sample diluted with 500 µl SDB/Na).
- If using an internal standard (e.g. norleucine) at this point of sample preparation dilute the sample in the same way but with the following changes.
  - Pour the same aliquot of filtered hydrolysis solution in a vial and add 10 µl of internal standard solution (e.g. norleucine) with a concentration of 10 µmol/ml and dilute with a by 10 µl reduced amount of SDB/Na (e.g. 500 µl sample add 10 µl internal standard solution diluted with 490 µl SDB/Na).
- Mix the solution for homogeneity (e.g. vortex mixer)
- These solutions are now ready for injection.

Sykam GmbH
Gewerbering 15
D-86922 Eresing

Telephone +49 (0) 8193/93820
Telefax +49 (0) 8193/938220

Internet www.sykam.com

Additional Information:

- In case of massive retention time variations in the beginning of a sample chromatogram compared to a standard measurement check the adjustment of the pH value after dilution with SDB/Na. The pH value should be in the range of about 2.10 to 2.30.
- Due to the decomposition degradation of all amino acids except tryptophan during this kind of hydrolysis it would be required to use different sample preparation procedures for the determination of other proteinogenic amino acids. Therefore the acidic hydrolysation with or without forced oxidisation can be used. Generally it is nearly impossible to avoid oxidisation processes of the oxygen sensitive amino acids methionine and cystine. Due to this the quantitative determination of these amino acids is limited. To achieve approximate results for these amino acids without a forced oxidation it is important to reduce the oxygen concentration in the headspace of the hydrolysation vessel as good as possible. In case that these amino acids are not of higher interest the simple acidic hydrolysation procedure can be used (see SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159). If reliable quantitative results of aforementioned amino acids are required the acidic hydrolysis with forced oxidation should be applied (see SOP: Protein sample preparation by acidic hydrolysis with oxidisation, Sykam QM-4160).
- If the time period between sample preparation and measurement is longer than a few hours, the prepared sample should be store at 2-8 °C or colder. The dilution factor for the described sample preparation depends on the following preparation steps:
  - mass change factor after drying
  - mass change after defatting
  - sample amount used for hydrolysis
  - dilution by oxidation solution
  - dilution by hydrolysis solution
  - if applied dilution by internal standard addition before hydrolysis
  - dilution after pH adjustment
  - factor of dilution for injection (including ISTD if applied)

References:

[1] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed (Text with EEA relevance)

[2] SOP: Protein sample preparation by acidic hydrolysis, Sykam QM-4159).

[3] SOP: Protein sample preparation by acidic hydrolysis with oxidation, Sykam QM-4160).

[4] SOP: Preparation procedure for Amino Acid Standard Additions QM-4101