SYKAM CHROMATOGRAPHY

STANDARD OPERATION PROCEDURE

Protein sample preparation by acidic hydrolysis

Draft

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Index

1. Version History

Modification correlated to last version should be indicated in document.

Date	Status / Version	Author	Modifications
18.03.2016	0.1	Jasper Hölzer	Created

2. Scope of Application

Sample preparation of proteins and peptides by acidic hydrolysation (with limitations for methionine and cystine and without tryptophan)

3. Description

This procedure describes the preparation of protein and peptide containing samples by acidic hydrolysation for the qualitative and quantitative determination of amino acids.

For the analysis of the proteinogenic amino acids it is required to dissociate the amino acid polymer to single amino acids. The proteins and peptides are digested completely by hydrolysing the peptide bond between two amino acids catalysed by hydrochloric acid.

For reliable quantitative results it is important that the sample preparation procedure is strictly complied regarding reaction times and temperatures.

During the hydrolysis it is nearly impossible to avoid oxidation processes which will transduce oxygen sensitive amino acids methionine and cystine into their oxidized derivatives methionine-sulfone and cysteinic acid. To reduce this effect as good as possible the headspace in hydrolysation vessel must be free of oxygen. Due to this the quantitative determination of the aforementioned amino acids is limited. For a reliable result use the oxidative hydrolysis procedure (see SOP: Protein sample preparation by acidic hydrolysis with oxidation, Sykam QM-4160).

Related to the acidic media used for hydrolysis, amino acids such as asparagine and glutamine will be transduced into the acid derivatives aspartic acid and glutamic acid.

Tryptophan is not stable to persist the acidic hydrolysis and will decompose completely during hydrolysation. If the concentration of tryptophan has to be determined, it will be required to perform additionally a basic hydrolysation (see SOP: Protein sample preparation by basic hydrolysis, Sykam QM-4161). On the other hand the basic hydrolysation will degrade all other amino acids.

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.

4. Required Resources

4.1 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) with maximum volumes of 1000 µl, 200 µl and if internal standard should be used also 10 µl
- oven, block thermostat or similar heating device for hydrolysis vessels
- pH meter incl. pH probe and temperature sensor
- magnetic stirrer
- PTFE coated magnetic stir bar
- PTFE coated stir bar catcher
- vortex stirrer for micro centrifuge tubes and vials
- centrifuge** for micro centrifuge tubes, volume: 1.5 ml, rotation speed: ≥14.000 rpm
- refrigerator at 2-8 °C or similar cooling device

4.2 Chemicals

- deionized water or higher grade (conductivity ≤ 0.5 µS)
- hydrochloric acid, (e.g. 32 w/v%), analysis grade or higher
- phenol, analysis grade or higher
- sodium hydroxide, analysis grade or higher
- amino acids used as internal standard (ISTD) (e.g. D,L-Norleucine, CAS: 616-06-8)

4.3 Consumables

- disposable syringes, volume: ≥ 5 ml
- disposable syringe filters, pore size: ≤ 0.45 µm
- tips for micro pipets in required sizes
- sample vials, glass, screw cap with perforated Silicon/PTFE septum (Sykam Cat. No.: S000212)
- Sykam Sample Dilution Buffer for hydrolysate samples (SDB/Na) (Sykam Cat. No.: S000020)
- Amino acid standard mix for hydrolysis
  - Sykam Standard Type H: 18 amino acids (concentration in µmol/ml)
    Hydrolysate Standard with unit µmol/ml (Sykam Cat. No.: S000029)
  - Sykam Standard Type H-G: 18 amino acids (concentration in µg/ml)
    Hydrolysate Standard with unit µg/ml (Sykam Cat. No.: S000035)

* one per sample which are prepared simultaneously

** if alternative sample post preparation procedure is used

5. Procedure

5.1 Pre-preparation

1. Hydrolysis Solution

   Prepare a solution of 6 M hydrochloric acid with a concentration of 0.1 w/v% Phenol. Therefore fill 590 ml hydrochloric acid (32 w/v%, 1.16 kg/l, MW_{HCl} 36.46 g/mol) and 1 g Phenol in a 1 litre volumetric flask and fill up to 1 litre with deionized water.

2. Neutralization Solution 1

   Prepare a solution of 7.5 M sodium hydroxide in water. Therefore weigh in 300 g sodium hydroxide (MW_{NaOH} 40.00 g/mol) in a 1 litre volumetric flask and add about 500 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.

3. Neutralization Solution 2

   Prepare a solution of 1 M sodium hydroxide in water. Therefore weigh in 40 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.

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.

5.2 Sample Pre-preparation

- 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 till weight remains constant or freeze dry,
  - determine the weight loss during drying for later calculations if required.
- If the sample material has a high lipid content (> 5 %)
  - weigh in a few gram or less of dry sample,
  - reduce the lipid 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.
- Use representative subset of the sample and homogenize it by grinding till the particle size is approximately ≤ 0.5 mm.
- 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.

5.3 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 25 ml of Hydrolysis Solution (preparation see above subsection: Pre-preparation)
- If using an internal standard at this point of sample preparation add 1000 µl of internal standard solution (e.g. norleucine) with a concentration of 4 µmol/ml to the sample.
- Mix the sample carefully with the hydrolysis solution by swivelling.
  - Take care that 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.
  - 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.

5.4 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 to observe the temperature of the hydrolysis solution during pH adjustment.
- Add slowly about 18 ml of Neutralisation Solution 1 (7.5 M sodium hydroxide solution).
  - Thereby the temperature must 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.
- Alternative pH adjustment (simplified but not that precise, retention times may vary)
  - Add slowly about 18.5 ml of Neutralisation Solution 1 (7.5 M sodium hydroxide solution).
  - Thereby the temperature should not exceed 40 °C.
- 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 it 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.
- If the sample is not measured within a few hours, store the graduated flask at a temperature of 2 to 8 °C until usage.
- 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 milliliter of the solution!
  - Alternative: Take an aliquot of the hydrolysed sample solution and transfer it to a centrifuge tube and centrifuge solution at 14.000 rpm for 10 minutes.
- Dilute the filtered 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).
- These solutions are now ready for injection.

6. 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 2.00 to 2.20.

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
- Factor of dilution for injection (including ISTD if applied)

In Case of ambiguities don't hesitate to contact labsupport@sykam.com

7. Literature

[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 with oxidation, Sykam QM-4160

[3] SOP: Protein sample preparation by basic hydrolysis, Sykam QM-4161

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