STANDARD OPERATION PROCEDURE
Faculty of Biosciences, NMBU

Method name: Tryptophan
BIOVIT No: Arb1051

---

1. Introduction

The method determines the total tryptophan content of feed and faeces, by basic hydrolysis, separation by liquid chromatography (HPLC) and fluorometric detection. The method does not distinguish between D and L- tryptophan.

2. Reagents

- 10.5 M NaOH: 420 grams of NaOH are dissolved in H_2O (MilliQ) to a total of 1 liter
- 6 M HCl: 499 mL HCl (37%, fuming) is diluted in H_2O (MilliQ) to a total of 2 liters
- 0.5 M Na_2HPO_4 (x $2H_2O$): 44.5 grams are dissolved in 1 M HCl to a total of 500 mL
- Methanol w/1% (w/v) 1,1,1-trichloro-2-methyl-2-propanol (TCMP): 10 grams of TCMP are dissolved in methanol to a total of 1 liter
- 0.5 M ortho-phosphoric acid: 34 mL diluted with H_2O (MilliQ) to a total of 1 liter

Buffer for HPLC:
1 liter: 3 grams acetic acid (100%), 900 mL H_2O (MilliQ), 50 mL methanol with 1% TCMP, adjust pH to 5.0 with ethanolamine, make up to 1000 mL with H_2O (MilliQ) and filter with membrane filter (589 whatman black ribbon).

2 liters: 6 grams acetic acid, 1800 mL MilliQ-H2O, 100 mL methanol with 1% TCMP, adjust pH to 5.0 with ethanolamine, make up to 2000 mL with H_2O (MilliQ) and filter with membrane filter (589 whatman black ribbon).

2.50 mM D, L-tryptophan solution (*tryptophan stock solution*)

100 mL:

- 51.06 mg D, L.-tryptophan (write down the exact weight with two decimals)
- 70 mL 0.1 M NaOH
- 2 mL 0.5 M Na_2HPO_4 solution
- 5.6 g NaCl
- Adjust the pH to 6.2 with HCl
- Fill up to 100 mL with H_2O (MilliQ)
- Freeze at -80 ° C in 5 mL aliquots

2.50 mM internal standard solution

100 mL:

- 54.56 mg α-methyltryptophan (write down the exact weight)
- 70 mL 0.1 M NaOH
- 2 mL 0.5 M Na_2HPO_4 solution
- 5.6 g NaCl
- Adjust the pH to 6.2 with HCl
- Fill up to 100 mL with H_2O (MilliQ)
- Freeze at -80 ° C in 5 mL aliquots

Preparation of Standard 1-2-3 (with tryptophan and internal standard)

	STD 1 25 µM	**STD 2 50 µM**	**STD 3 75 µM**
	50 mL:	50 mL:	50 mL:
NaCl	3,25 g	3,25 g	3,25 g
H_2O (MilliQ)	35 mL	35 mL	35 mL
0,5 M Na_2HPO_4- solution	2,5 mL	2,5 mL	2,5 mL
2-propanol	5 mL	5 mL	5 mL
2,5 mM-tryptophan solution	0,5 mL	1 mL	2 mL
2,5 mM internal standard solution	0,5 mL	1 mL	2 mL

- Adjust the pH to 6.2 with NaOH
- Make up to 50 mL with H_2O (MilliQ)
- Freeze at -80 ° C in 0.5 mL aliquots

3. Risk assessment
Wear lab coat, goggles and gloves (nitrile) when making HCl and NaOH solutions. If you get concentrated HCl or NaOH on your skin, rinse with large amounts of water. Weigh barium hydroxide octa-hydrate in fume hood with gloves (risk of skin burns). Toxic by inhalation: use face mask with P3 filter when opening heating cabinet.

4. Equipment
- Ultimate 3000 UHPLC with auto-injector and fluorescence detector
- pH meter
- Heating cabinet (110 °C)
- Whirl mixer
- Table centrifuge
- Polypropylene (PP) bottle, 125 mL, wide neck and screw cap
- Scintillation glass
- 150 mL beaker
- 100 mL volumetric flask
- Funnel
- 2 mL GC vials
- 0.3 mL GC vials
- 0.45 µm membrane filter
- 2 mL disposable syringe

5. Special remarks
Protect standard solution and hydrolysates from direct sunlight.

Samples with a high fat content (> 40%) must be extracted with petroleum ether (bp 40-60) before the hydrolysis.

The finished hydrolyzed samples have a short shelf life and must be analyzed within 3 days. Freeze a small portion of the sample at -80 ° C in case of re-analysis.

6. Sample material
The amount of sample material is calculated from the amount of nitrogen in the samples. This is first determined by the Kjeldahl-N method (alternatively dumas-N).

$$\frac{10\,mg}{amount\ of\ nitrogen\ (\frac{g}{kg})} \times 1000\ (\frac{g}{kg}) = sample\ to\ be\ weighed\ (mg)$$

Amount of sample: 100 - 1000 mg homogeneous sample (samples should contain about 10 mg nitrogen).
Degree of grinding: 0.5 mm.

Faeces: 1 parallel
Feed: 3 parallels (recommended)

7. Job description

1. Weigh the sample in a PP hydrolysis bottle
2. Add 8.4 g of barium hydroxide octahydrate
3. Add 8 mL of degassed H_2O (MilliQ)
4. Mix on a vortex or magnetic stirrer. Keep a Teflon-coated magnet in the solution
5. Add 2.00 mL (2.5 mM) internal standard solution
6. Wash any sample from the walls of the bottle with 4 mL of water
7. Put the cap on loosely and place the bottle in a preheated heating cabinet (110 ° C)
8. Incubate for 1 hour, tighten the cap and continue to hydrolyze for 19 hours (110 °C)
9. Put the samples in a fume hood and carefully unscrew the caps. If it starts to bubble in the bottle - screw the cap back on and wait a few seconds. Do not wait too long before removing the cap - otherwise the bottles will collapse.
10. Add 30 mL of warm H_2O (MilliQ) and put the cap on again
11. Shake or stir lightly
12. Cool the bottles in ice bath for 15 min
13. Add 5 mL of ortho-phosphoric acid (0.5 M)
14. Transfer the hydrolyzed sample to a labeled beaker - keep the beaker on ice
15. Rinse the bottle with about 20 mL H2O (MilliQ)
16. Add 20 mL of methanol
17. Adjust the pH to 6.2 with 6 M HCl
18. Transfer the sample to a volumetric flask and dilute to 100.0 mL
19. Label a scintillation glass and feel up with the sample
20. Freeze a small portion of the sample at -80 ° C

21. Filter the sample with 0.45 membrane filter + disposable syringe
    - discard the first 10 drops
    - fill up a 2 mL glass vial for the HPLC analysis.
22. Pick up standard 1-2-3 from the freezer and transfer to 0.3 mL GC vials.

HPLC analysis

- Column: Supelcosil LC-18 (4.6 mm, 15 cm, 5 µm)
- Column temperature: room temperature
- Mobile phase: 92% buffer: 8% methanol (See details under «reagents»)
- HPLC system: Ultimate 3000 UHPLC system with autosampler (Thermo Scientific)
- Detector: RF-535 fluorescence detector (Shimadzu)
- Flow rate: 1 mL/min
- Total analysis time: about 25 min per sample
- Wavelengths: excitation: 280 nm, emission: 356 nm
- Injection volume 30 µL
- Software: Chromeleon

23. Open the chromeleon and find the instrument side of the ultimate 3000.
24. Purge all four channels (otherwise there may be air in the system)
25. Set Channel A to 92 % and Channel B to 8 %
26. Set flow to 1 mL/min
27. Turn on the fluorescence detector (two buttons on the left side)
28. Wait about 30 min
29. Reset the fluorescence detector manually (front wheel) and check that the baseline is stable.
30. Put the vials with standards and samples in the autosampler.

Setting up a sequence in Chromeleon

31. Open Chromeleon - IHA-SUMMIT \ ChromeleonLocal - Instrument data - Ultimate 3000 - sequence - tryptophan
32. Copy a previous sequence (right-click + copy, and paste under the desired folder)
33. Make the sequence look like this (adjust to the number of samples):
    - Blank (methanol)
    - Standards 1-3
    - Control sample
    - Samples
    - Standards 1-3
34. Fill in the correct name and type (unknown = samples, calibration standard = standard)
35. Insert level «2» for std1, level «3» for std2 and level «4» for std 3
36. Check that the method is called "tryptophan_hanne_std"
37. Enter the same position as the samples in the autosampler (for example BA1 for the first hole on the left in rack B.
38. Mark all the samples in the sequence (everything should be black).
39. Press Ctrl + c / Ctrl + v - (goes from «finished» to «idle»).
40. Give the sequence the following name; YYMMDD_rekvXX_lastname.
41. Press «start»
42. If the analysis is to go overnight, a line with the "shutdown" method can be added at the end. Remember to set the position to a hole with a vial (for example the blank sample), otherwise the analysis will be interrupted, and the pump will continue and run = will run out of mobile phase and draw in air!!

8. Calculation

- Open the chromatograms and check that the baselines are laid correctly and that the peaks have come under the correct "tag" (TRP or Met-TRP)
- Check the calibration curve for the standards of TRP and Met-TRP
- Go to "interactive results" and retrieve the results under "amount fluorescence TRP" and "amount fluorescence Met-TRP".
- Paste these numbers into the excel sheet "calculation sheet" below:
  - labmal- various analyzes - tryptophan
- Check that the correction is for the internal standard in use (check date for IS)
- Enter weights, nitrogen content and name for all samples
- The sheet now calculates automatically
- Remember to save the excel sheet on the form; YYMMDD_rekvXX_lastname