**STANDARD OPERATION PROCEDURE**

**Faculty of Biosciences, NMBU**

**Method name: Amino acid analysis (oxidized samples)**

BIOVIT No: Arb1050

**1. Introduction**

The method determines the total content (peptide-bound and free) amino acids in feed and faeces, using an amino acid analyzer with ion exchange column, post-column derivatization and photometric detector. The method is suitable for the amino acids cyst (e) in, methionine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine (NOT tryptophan).

**2. Reagents**

* Oxidizing solution - stored in the refrigerator.
* Measure out 111 mL of H2O (MilliQ) and add to a 1 L flask kept in an ice bath
* Add, carefully, 750 mL formic acid (98-100%)
* Add 4.73 g of phenol and mix well
* Hydrolysis solution - stored in the refrigerator.
* Measure out 400 mL H2O (MilliQ) and add to a 1 L flask kept in an ice bath
* Add, carefully, 492 mL HCl (37%)
* Add 1 g phenol and make up to 1 L with H2O (MilliQ)
* 7.5M NaOH
* Weigh out 300 g NaOH and dissolve it in portions in 500 mL H2O (MilliQ)
* Use magnetic stirring
* Make up to 1 L with H2O (MilliQ) when the solution has cooled down
* Hydrogen peroxide (30%)
* Sodium disulfite (sodium metabisulfite)
* Weigh 0.84 g into scintillation glasses with lids
* Loading buffer (Sodium Citrate Loading Buffer pH 2.20)
* Control sample: soya control

|  |  |  |
| --- | --- | --- |
| **Buffers for Biochrom30** | **Order number** | **Buffer number** |
| Sodium Citrate Loading Buffer pH 2.20 | 80-2037-67 | LoadingBuffer |
| Sodium Citrate Buffer pH 3.35 | 80-2037-80 | 1 |
| Sodium Citrate Buffer pH 4.25 | 80-2038-08 | 2 |
| Sodium Citrate Buffer pH 2.65 | 80-2037-79 | 3 |
| Borate / Citrate Buffer pH 8.60 | 80-2037-88 | 4 |
| Sodium Hydroxide Solution | 80-2037-57 | 6 |
| Ultra Ninhydrin Regent Kit | 80-2118-30 | Ninhydrin |

**3. Risk assessment**

* Wear safety goggles, gloves and work in fume hood when handling phenol and acids.
* Use weight in a fume hood when weighing in sodium disulfite.
* Boiling of phenolic solution MUST be carried out in a heating cabinet WITH ventilation. When unscrewing the caps, the heating cabinet must be switched off BEFORE the door is opened and a face mask (P3), goggles and heat-protective gloves must be used. Put notes on both doors into the room that no one should enter while the samples are boiling.

**4. Equipment**

* Biochrom 30+ Amino Acid Analyzer
* Alias ​​autosampler
* Heating cabinet (110 °C)
* Whirl mixer
* Table centrifuge
* Test glass 60 mL
* Scintillation glass
* 150 mL beaker
* 200 mL volumetric flask
* Funnel
* 2 mL GC vials
* 0.3 mL GC vials
* 0.20 µm membrane filter (alternatively 0.45 µm)
* 2 mL disposable syringe

**5. Special remarks**

If cysteine ​​and methionine is of no interest, the oxidation step can be skipped to save time.

It is possible to use the method to look at other amino acids than those mentioned in the reference method (1). Examples: taurine, hydroxyproline and GABA (needs another standard).

Tyrosine will degrade a bit during oxidation. This is corrected for in the spreadsheet, however if the levels of tyrosine are abnormally low, there may have been more degradation than usual.

It is important that the samples do not stand for oxidation longer than described in the method!

The method used to analyze amino acids causes a deamination of the two amino acids Asparagine and Glutamine, and they are turned into Aspartic acid and Glutamic acid.

So:

glutamic acid = glutamic acid + glutamine

aspartic acid = aspartic acid + asparagine

When publishing this may be clarified by writing: Glx and Asx

(Glx= Glu + Gln) (Asx = Asn + Asp)

Or write: “Glu + Gln” or “Asn + Asp

**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)

Amount of sample: 100 - 1000 mg homogeneous sample (samples must contain about 10 mg nitrogen).

Degree of grinding: 0.5 mm.

Faeces: 1 parallel

Feed: 3 parallels (recommended)

**7. Job description**

Weighing of samples

1. Calculate the amount of sample to be weighed (done in the requisition sheet)
2. Weigh the calculated amount of sample in test tubes, 60 mL (with red cap)
3. Remember to include a control sample

Oxidation

1. Make the oxidation mixture:
   * 4.5 mL oxidizing solution x number of samples.
   * 0.5 mL hydrogen peroxide (30%) x number of samples.
   * Cover the container and let the mixture stand for 1 hour at room temperature
2. Add 5 mL of oxidation mixture to the samples.
3. Mix only if absolutely necessary (small lumps will dissolve on their own).

***NB! try to get as much of the sample material as possible into the glass, not the edge.***

1. Incubate the samples at 4 ° C in the refrigerator for 17 hours. (Ex.: in 15.00 - out 08.00.)
2. After 17 hours, the samples are taken out and 0.84 g of sodium disulfite is added to all the samples (weighed in advance in scintillation glass with screw cap)

Hydrolysis

1. Set the heating cabinet to 110 °C.
2. Add 25 mL of hydrolysis solution to the samples. Work in fume hood.
3. Put the caps loosely on
4. Set the samples in the heating cabinet (110 ° C).
5. After 1 hour, screw the caps on tightly. *NOTE: turn off the heating cabinet before opening the door + wear a mask and gloves. (Phenol = toxic)*
6. Continue the hydrolysis/boiling for 23 hours.

Transfer and pH adjustment

1. Take the samples out of the heating cabinet and cool them in ice water in a fume hood
2. Release the pressure by opening the caps and leaving the samples on ice for 15 min.
3. Label 150 mL beaker with sample number.
4. Transfer cooled sample to beaker
5. Rinse out of the test tube three times with loading buffer
6. Dilute the sample with loading buffer until the volume is approx. 75 mL.
7. Put the beaker in an ice bath
8. Add 10 mL of 7.5 M NaOH to all samples and cool for at least 15 min
9. Calibrate the pH meter according to the manual
10. Adjust the pH in the samples to between 2.0 and 2.4 with 7.5 M NaOH.
11. If the pH becomes too high, a drop of 6M HCl can be added

Dilution of pH-adjusted samples

1. Label scintillation glasses with sample number
2. Transfer the sample to a 200 mL volumetric flask.
3. Rinse out the beaker with loading buffer.
4. Dilute to 200 mL with loading buffer.
5. Mix well (turn the flask up/down 12 times).
6. Pour off some of the sample and then fill up a Labeled scintillation glass with sample.
7. Pour the rest into the sink.
8. Rinse the volumetric flask with water (RO) and use it again.

Preparation of samples and standards

1. Filter about 2 mL of the prepared samples (discard the first 10 drops) and transfer the filtered solution to 2 mL glass vials. Use syringe and syringe filter (0.2 µm).
2. Cork the glasses.
3. Pick up four standards (100, 200, 300 and 400 mM) in the -80 ° C freezer
4. Mix the standards on the whirl mixer. Transfer to 0.3 mL glass.
   * Make sure there are no air bubbles in the bottom of the glasses.
5. Cork the glasses.

Preparation of Alias ​​autoinjector

1. Check that there is enough washing solution (20% isopropanol in H2O (MilliQ)) in the bottle to the left inside the auto-injector.
2. Open "Alias ​​manager" on the desktop
3. Check that the temperature control is on and that it is at 4 ° C
4. Wash injector: WASH CONTROL – START (Repeat at least 2 times)
5. Set standards and samples in autosampler:
   * Open the front cover and carefully remove the Styrofoam lid
   * Press FRONT (under tray control) = tray comes out
6. Set Standards in position 1-4. Write down the order of the samples.
7. Press HOME = tray enters
8. Carefully attach the Styrofoam lid

*NB: If the autosampler does not cool - look for ice formation on the fan*

Launch Biochrom 30

1. Check status of ninhydrin & buffer solutions, fill up if necessary (see separate section)
2. Open the "BioSys Manual" on the desktop
3. Check LAMP and REACTION COIL (Press «reaction coil» enter 135 °C / Press «*column temp*» and enter 50 °C)
4. When Column temp has reached 50 °C, start conditioning with buffer 3.
5. When the reaction coil has reached 135 °C, start the Ninhydrin pump.
6. Look at the pressure that all the 3 black lines go up. If the pressure on the ninhydrin drops- check the connections around the right ventricle (remember orange gloves), most likely there is air in the system, especially if the ninhydrin has been refilled.
7. Check that the baseline for the two channels is stable (monitor baseline in Chromeleon)

Refilling of buffer/ninhydrin on Biochrom30

1. Check that there is enough buffer to run all the samples (especially nr 3 & 4).
2. Fill up if necessary. Remember to fill about 150 mL extra so that the tubes in the bottles do not draw air at the end.
3. Check that there is enough ninhydrin solution.
4. If not: Make a new one and fill up (SOP included in the box).
   * NEVER fill up during an analysis!
   * Remember to change the filter: first put this in a beaker with ninhydrin solution and keep in an ultrasonic bath for 5 min to get air out, if time: leave in ninhydrin overnight).
5. Check that the waste container is not full - empty if necessary (in the sink).

Set up sequence in Chromeleon

1. Open Chromeleon.
2. Copy a previous sequence (right-click + copy, and paste under the desired folder)
3. Make the sequence look like this (adjust to the number of samples):
   * Standards (1-4)
   * Control sample
   * 10 samples
   * Standards
   * 10 samples
   * Standards
4. Mark all the samples in the sequence (everything should be black).
5. Press Ctrl + c / Ctrl + v - (goes from «finished» to «idle»).
6. Save the sequence; YYMMDD\_rekvXX\_lastname.
7. Press «start»
8. Chromeleon is now ready for analysis and is waiting for a signal from BioSys.
9. Must stand as RUNNING.

Set up sequence in BioSys

1. Open the BioSys Manual.
2. Press *View* and *Program Control.*
3. Press *Insert.*
4. Press *Program Filename* and open the folder «Standard programs»
5. Select: USE Sodium Oxidized Hydrolysate.prg
6. Select sample ID. (rekv number + name)
7. Press No.Samples: enter the number of analyzes (check the number of injections in chromeleon).
8. Press *Vial no.* And enter where the first test / standard starts.
9. Press *Volume* and enter 40 μL.
10. Press *Details* and check that *Partial loop fill injection* is checked.
11. Then press *OK* and *OK.*
12. The very first sample in the BioSys sequence should be called "regen". This must be included as the column material must be regenerated before the first injection.
13. Select the first line and press "insert" - a new line will appear.
14. This must have its own program: USE Sodium Oxidized Hydrolysate regen.prg
15. Be sure to enter the correct "vial no (s)" for the standards and samples to be analyzed several times (must be entered manually).
16. Check that the sequence in Biochrom, after "regen", is identical to the sequence in Chromeleon, both name and location.
17. Write sample number + vial number in the BioSys book.
18. Press *Save as.* (Save under *Sample Lists*)

Start the analysis

1. Open BioSys Programs: 1000.
2. Highlight "regen" and press **RUN.**
3. Chromeleon should not start recording until standard 1 is injected
   * autosampler sends signal – check that this happens
4. Monitor that no air has entered during the analyzes (will give deviating retention times)
5. Biochrom30 turns off temperatures and pumps when the analysis is complete.
6. Uncheck **LAMP** if the instrument is not to be used for a couple of days

**8. Calculation**

* Open all the chromatograms and check that the baselines are laid correctly and that the peaks have entered under the correct name
* Check the calibration curves for the standards of all amino acids
* Go to "interactive results" and extract all the areas
* Paste these numbers into the excel sheet «Calculation sheet\_AA» which is under:
  + labmal- diverse analyser - Amino acids
* Enter weights, nitrogen content and names for all the samples in all the tabs in the excel sheet
* The sheet now automatically calculates g/kg of all amino acids

*NB: Separate sheet for water-corrected results!*

* Remember to save the excel sheet on the form; YYMMDD\_rekvXX\_lastname

***Reference***

1. Commission Regulation (EC) No 152/2009. 27 Jan 2009. Laying down the methods of sampling and analysis for the official control of feed. Annex III, P, Official Journal of the European Union L54 / 1 from 26/02/2009