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## Amino Acid Analysis Service

Product	Cat#	Package size
Amino Acid Analysis of given sample (total hydrolysis)	P2145.0001	Set-up
Amino Acid Analysis of given sample (without total hydrolysis)	P2145.0002	Set-up
Amino Acid Analysis from animal food	P2145.0003	Set-up
Amino Acid Analysis from physiological sample	P2145.0004	Set-up

### Service description

The amino acid analysis service encloses the total hydrolysis of a given sample, the chemical derivatisation of the free amino acids (either produced by the acid hydrolysis or of already free amino acids), the separation of the derivatised amino acids by reversed phase HPLC and subsequent interpretation of the result.

In general pure samples are required. The presence of salts, buffers, or detergents is deleterious. Amines (primary or secondary) will react with the carbamate, adversely affecting results. While salts, especially strong buffer substances can alter the pH of the sample causing the hydrolysis/derivitisation to be incomplete or simply fail. Additionally, significant levels of glycerol or carbohydrates are problematic - the glycerol is nonvolatile and attracts moisture (acid) and carbohydrates char, decomposing to ash taking the sample with them. If it is impossible to clean up your sample, we can attempt other desalting methods, such as precipitation or reverse phase HPLC cleanup.

For analysis we do need at least 25.0µg (better 50.0µg) (500pmoles to 1000pmoles) protein, based on an average molecular weight of 25,000 Da. For peptides, the requirement is much smaller - 5.0µg to 20.0µg (5nmol to 10nmol).

Delivery times for results are in the range of about 7 working days, but may vary due to demand.

Samples can be send by post or parcel service to Genaxxon bioscience GmbH. Samples can be dry or in liquid (most preferable in low salt buffer or better in demineralised water). Please make sure that your sample is packed safely and can not break or leak.

Please support us with information about the composition of your sample(s) to enable us to act accordingly.

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## Description of amino acid analysis

Amino acid analysis is used to determine the quantity of each individual amino acid in a given sample (protein - peptide - food, blood, serum, urine or other matrix). For being able to analyse a given sample there are four principle steps in amino acid analysis:

1. Hydrolysis of given sample (if necessary)
2. Derivatisation (chemical modification of free amino acids to enhance the detection limit)
3. Separation of derivatised amino acids (chromatographic separation (thin layer or HPLC of derivatised amino acids).
4. Data interpretation and calculations

- **1a. Hydrolysis (e.g. pre-column derivatisation with FMOc or PITC)**

A known amount of internal standard is added to each sample (Since norleucine does not naturally occur in proteins, is stable to acid hydrolysis and can be chromatographically separated from other protein amino acids, it makes an excellent internal standard). The molar amount of internal standard should be approximately equal to that of most of the amino acids in the sample. After the addition of norleucine the sample, containing at least 10nmoles of each amino acid, is transferred to a hydrolysis tube and dried under vacuum (The estimated amount of protein required for an accurate analysis is at least 10.0µg (better 20.0µg) (200pmoles to 400pmoles), based on an average molecular weight of 25,000 Da. For peptides, the requirement is much smaller - 1.0µg to 2.0µg (1nmol to 2nmol), based on an average molecular weight of 1,000 Da)). The tube is placed in a vial containing 6N HCl and the protein is hydrolyzed by the HCl vapors under vacuum. The hydrolysis is carried out for 65 minutes at 150 °C. Following hydrolysis, the sample is dissolved in distilled water containing EDTA. Hydrolysis can have varying effects on different amino acids (see Table).

- **1b. Hydrolysis (post-column derivatisation with Ninhydrin)**

An aliquot of each sample is transferred into a separate hydrolysis glass tube and supplemented with 300µL 6N HCl, sealed under vacuum (<20mbar) and hydrolysed for 24h at 110 °C. After hydrolysis each sample is dried at 36 °C for 8h (Vacuum centrifuge).

Each dried sample is supplemented with an adequate volume of sample buffer (Na-Acetate buffer, pH2.2) for subsequent derivatization and HPLC chromatography.

- **2a. Derivatisation (pre-column derivatisation)**

The free amino acids cannot be detected by HPLC unless they have been derivatised. Derivatisation has to be performed before (pre-column derivatisation) or after the HPLC run. In pre-column derivatisation, free amino acids react, under basic conditions, e.g. with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acid derivatives. This process takes approximately 30 minutes per sample. Following derivatisation, a methanol solution containing the PTC-amino acids is transferred to a narrow bore HPLC system for separation.

- **2b. Derivatisation (post-column derivatisation)**

In post-column derivatisation amino acids react after their separation via a cation exchange column in a reactor with Ninhydrin to produce a coloured derivative that can be detected with a photometer. A standard solution containing a known amount (e.g. 200pmol) of 20 common free amino acids is also loaded, separated and derivatized. This will be used to generate a calibration file that can be used to determine amino acid content of the sample.

- **3a. HPLC separation (pre-column derivatisation)**

The PTC-amino acids are separated on a reverse phase C18 silica column and the PTC chromophore is detected at 254 nm. All of the amino acids will elute in approximately 25 minutes. The program is run using a gradient of 2 buffers.

- **3b. HPLC separation (post-column derivatization)**

A polymeric cation exchanger is used to separate amino acids by HPLC chromatography (particle size: 4µm; column dimensions: 125 x 4mm ID). Separated amino acids are detected by post-column Ninhydrin derivatisation at 125 °C and photometric measurement at 570nm. Sample volume (20µL) is applied via a sample loop.

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- **4. Data interpretation and calculations**

Chromatographic peak areas are identified and quantitated using a data analysis system that is attached to the amino acid analyzer system. A calibration file is used that is prepared from the average values of the retention times (in minutes) and areas (in Au) of the amino acids. Since a known amount of each amino acid is loaded onto the analyzer, a response factor ((Au/pmol) can be calculated. This response factor is used to calculate the amount of amino acid (in pmols) in the sample.

#### Some evaluation examples

- **Mole percent** represents the amount of each amino acid present as a percentage of the total amino acids recovered in the sample. Mole percent can be useful for samples in which there is no known composition or molecular weight, non-specific molecular weights, or the sample contains mixtures of proteins, free amino acids and other components.

$$[\text{pmol of individual amino acid}] / [\text{total pmol of all amino acids in the sample}] \times 100 = \text{mole percent of each amino acid}$$

- **Composition by molecular weight** can be used when the molecular weight of the sample is known and the amino acid composition is desired.

$$[\text{pmol of amino acid}] \times [\text{residue molecular weight of amino acid}] = \text{picogram of amino acid}$$

$$\text{sigma}[\text{picogram of all amino acids}] / [\text{pmol of sample recovered}] = \# \text{ of residues of amino acid per molecule of sample}$$

- **Composition by residue** is used when the amino acid composition is required and the number of times a particular amino acid residue occurs in the sample is known.

$$[\text{pmol of selected amino acid}] / [\text{known \# of residues of selected amino acid/sample molecule}] = \text{pmol of sample recovered}$$

$$[\text{pmol of amino acid}] / [\text{pmol of sample recovered}] = \# \text{ of residues of amino acid per molecule of sample}$$

#### Listing of some effects on amino acid analysis

##### Hydrolysis effects on different amino acids

Amino Acid	Effect
Valine, Isoleucine	Bonds are not easily broken
Threonine, Serine	Slowly destroyed by acid hydrolysis. Serine is a common contaminant.
Methionine	Partially oxidised during acid hydrolysis.
Asparagine, Glutamine	Converted to aspartic acid and glutamic acid
Tryptophane	Destroyed by acid hydrolysis
Cysteine	Destroyed by acid hydrolysis

## Listing of some effects on amino acid analysis (continued)

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**Effects of contaminants on derivatisation yield \***

Contaminant	Effect
Ammonium acetate	No adverse effect on results (only valid for pre-column derivatisation). Not possible for post-column derivatisation.
Sodium acetate	His low; Tyr, Val, Ile, Leu, Phe and Lys slightly lower
Triethylammonium acetate	His and Thr slightly lower (only valid for pre-column derivatisation). Not possible for post-column derivatisation
Ammonium bicarbonate	Thr slightly lower (only valid for pre-column derivatisation). Not possible for post-column derivatisation.
Sodium bicarbonate	His and Tyr low; Ile, Leu and Phe also low. (only for pre-column derivatisation). Not adverse effects for post-column derivatisation.
Sodium borate	No adverse effect on results
Sodium chloride	No adverse effect on results
Sodium phosphate	Low and variable yields of most amino acids (only for pre-column derivatisation). No problems with post-column derivatisation.
Triethylammonium phosphate	No adverse effect on results (only for pre-column derivatisation). Not possible for post-column derivatisation.
CAPS	Very large late elution peak obscures Phe and Lys with pre-column derivatisation.
HEPES	Artefact peak which co-elutes with Met
TRIS	His slightly low, artifact peak co-elutes with Tyr (only for pre-column derivatisation). Not possible for post-column derivatisation.
SDS	His and Thr slightly low, Cys and Lys yields are good
Triton X100	His and Thr slightly low, Cys and Lys yields are good

\* Effects of common buffer salts and detergents on amino acid derivatisation. All solutions were added to 100pmols of hydrolysate amino acid standard in a 20µL volume.  
 Concentration of salt solutions 50mM.  
 Concentration of detergent solutions 0.1% (v/v)

**Effects on metal ions on amino acid analysis results \*\***

Amino Acid	Effect
Aluminium	Asp and Glu very low, all other amino acids low except Pro
Boron	No significantly adverse effects, His and Thr may be slightly low
Copper	Cys and Lys almost gone, His low, Asp and Glu slightly low
Iron	Glu, Ser, His, Thr, Cys, Lys all low
Lead	Asp, Ser, Thr, Lys slightly low
Nickel	Almost no recovery of any derivatives except Thr and Pro
Zinc	Acidic and basic amino acids and Cys very low, Ser slightly low

\*\* All solutions were added to 100pmoles of hydrolysate amino acid standard in a 20µL volume  
 Concentration of solutions 20ppm