Gas Chromatography for Quality Control of Peptides – a Useful Tool

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Abstract:
Chromatography is an important tool for quality control of peptides. Beside the use of liquid chromatography, gas chromatographic methods also gives important information about purity and composition of a peptide matrix.

Measurement of the racemate content is an important aspect of the determination of the purity of peptidic products. Serveral analytical methods can be used: Optical rotation can indicate the presence of an epimeric impurity of the peptide. If the epimers are available, separation via liquid chromatography can often be achieved. Also NMR is capable of providing information about the configuration. One of the most accurate methods is determination of enantiomeric purity of the constituent amino acids after hydrolysis and deivatization using deuterated reagents followed by chiral capillary gas chromatography and mass selective detection.

With enantiomer labelling, accurate quantitative analysis of sensitive amino acids such as tryptophan, serine, methionine or tyrosine is possible. Due to the superior multi internal standards – the enantiomers – precision and accuracy of the amino acid analysis is excellent.

Beside determination of organic volatile impurities as a domain of gas chromatographic separation also quantitation of the counter ions acetate und trifluoroacetate is possible with high accuracy.

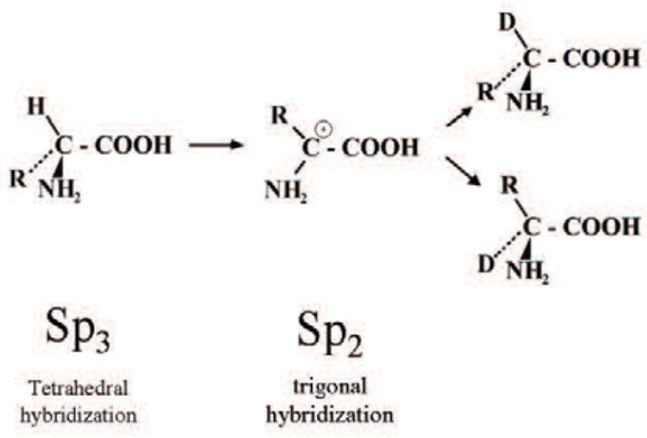
As an alternative to the Karl-Fischer method, the moisture content of peptides can be determined directly by thermal desorption of water and gas chromatographic separation from other volatile components.

Method of Determination of enantiomeric purity:

One of the most accurate and sensitive methods for the analysis of the optical purity of free amino acids involves derivatization and gas chromatographic separation of the enantiomers using a chiral stationary phase. A technique for the reliable quantitation of the racemate content of peptide- or protein-bound amino acids, eliminating the contribution of racemization during hydrolysis, was presented by Frank et al.[1] The protein or peptide is hydrolyzed in 6N DCl/D_2O, whereby racemization is accompanied by deuterium exchange in the α-C position. By mass spectrometric determination of the relative amounts of deuterium labeled to non labeled species of a characteristic ion, the proportion of racemate arising from the hydrolysis could be calculated. Using multiple ion detection, Liardon et al.[2] improved the methodology, deriving a series of equations with which the interference arising from neighbouring ions $(I+1)^+$ and $(I\ 1)^+$ could be taken into account. Using selected ions and deuterated derivatization reagents, a simplified version of the latter method was proposed [3] and its automation implemented.[4]
The method was validatetd for all proteinogenic and several non-proteinogenic amino acids.[5]

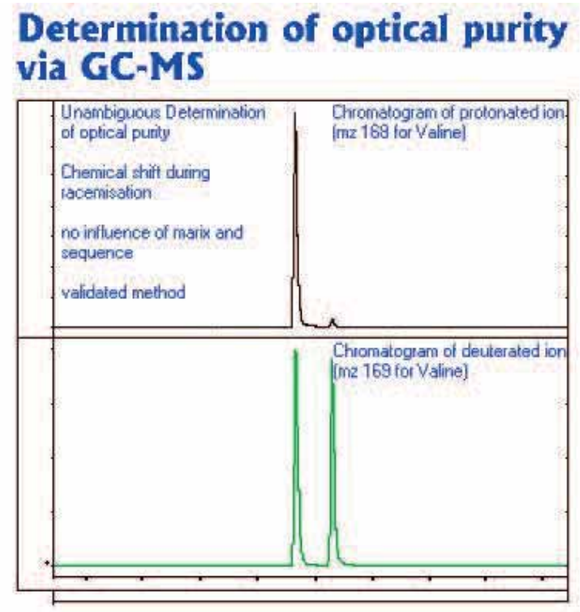
Racemization during acid hydrolysis involves protonation and de-protonation of the carboxyl group with tautomeric enol rearrangement at the α-C position. If the hydrolysis is carried out in a fully deuterated environment, the racemate formed is deuterium labeled at the α-position with a consequent shift of one mass unit of all fragments containing this moiety.

The proportion of D-amino acids originally present in the peptide is thus represented by the relative amounts of the unlabeled form.



Three prerequisites must be fulfilled:

1. It is imperative that the ion selected for monitoring $(I)^+$ includes the α-H.
2. The ion $(I-1)^+$ should be of low intensity (less than 5%) relative to the monitored ion $(I)^+$. Incorporation of deuterium would lead to its being detected together with the unlabeled ion $(I)^+$ and would result in a positive error with a value of the product of the relative intensity of $(I-1)^+$ and the degree of racemization during hydrolysis.
3. Interference from neighboring ions $(I+1)^+$ and $(I-1)^+$ could be taken into account using multiple ion detection using a series of equations.[2]



The validation comprises determination of the following parameters for each amino acid:
Precision (repeatability), Intermediate precision, linearity, accuracy, limit of quantitation, limit of detection and selectivity of the chromatographic system. It is demonstrated that in general the method is capable of reliably determining the optical purity down to 0.1% of the unnatural enantiomer. For certain amino acids such as cysteine and amino acids linked to cysteine, this value may increase to 0.3% in certain matrices.

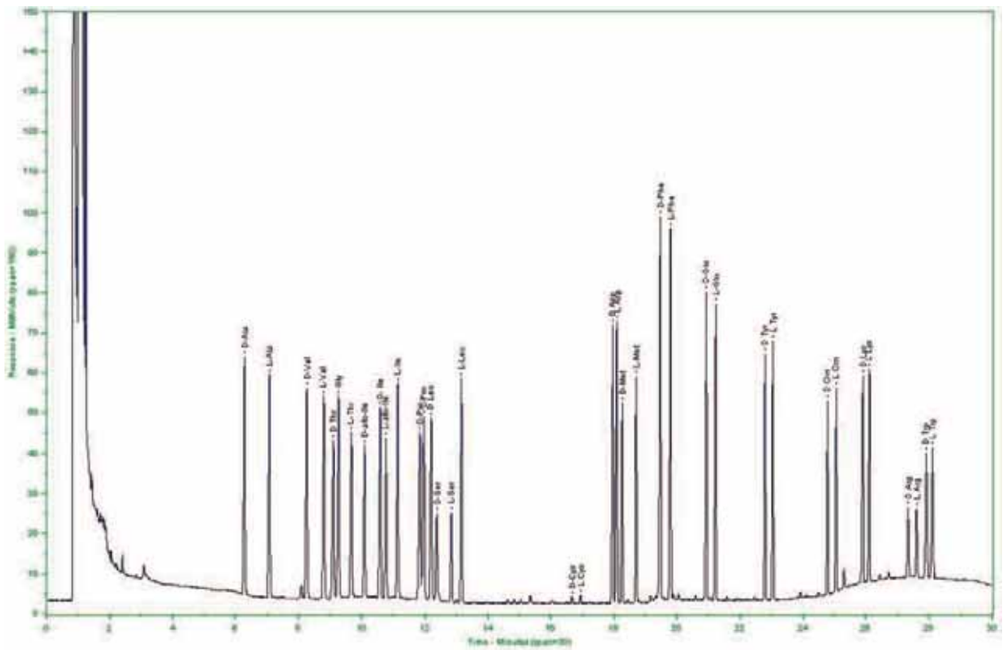
Quantitative amino acid analysis:

All methods for amino acid analyses use the internal standard method. A defined amount of a substance as chemically and physically similar as possible to the component to be determined is added to the sample, is co analysed, and is used as a basis for the calculation. The greater the similarity between the chemical and physical properties of the standard and the component to be determined, the more similar will be the discrimination in the analysis, and the better the analytical result. Often, more than one standard is used in order to achieve this. In addition, correction factors must be considered.
The method of enantiomer labeling[6] uses a standard that admirably fulfils these requirements: the enantiomer is added to an optically active substance. In the case of amino acid analysis this is a mixture of all amino acid enantiomers of defined composition. Each amino acid thus has its own standard the enantiomer. The separation is performed on an optically active phase which separates all these components. This is possible in capillary gas chromatography using Chirasil Val after derivatising amino acids to the n propyl esters and acylation with trifluoroacetic anhydride.

The fact that the optical enantiomer is a standard with not just similar, but identical physical and chemical properties in an achiral pool gives the following advantages:

- if the standard is added directly after sample collection e.g. immediately after collecting the blood sample for amino acid analysis in serum[7] then the losses of standard and sample during clean up, derivatization and sample injection are identical because these processes are performed in an achiral medium.
- The determination of amino acids, even in complex matrices, is simplified considerably because the methods used in clean up may be such that quantitative recovery of the amino acids is not necessary.
- Substance specific calibration factors need not be considered.

The enantiomeric purity of the sample as well as of the standard are to be taken into account.



Separation of the optical antipodes of proteinogenic Amino acids as N,O,S-TFA amino acid n-propyl esters on Chirasil Val

Calculation of the amino acid concentration:

$$\chi_\alpha = \frac{A_L - A_D \times C_D + A_L \times C_L - A_D \times C_L \times C_D}{A_D - A_L \times C_L + A_D \times C_D - A_L \times C_L \times C_D} \times mD$$

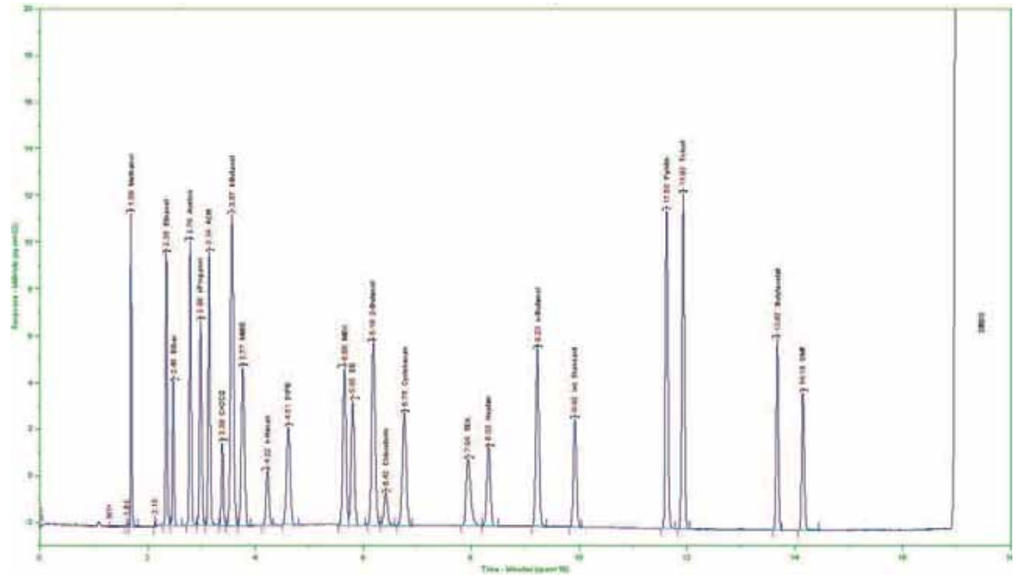
A_L	=	peak area of L enantiomer after addition of standard
A_D	=	peak area of D enantiomer after addition of standard
C_L	=	D enantiomer ÷ L enantiomer of the sample
C_D	=	L enantiomer ÷ D enantiomer of the standard amino acid
mD	=	amount of standard amino acid added
χ_α	=	amount of amino acid being determined

Determination of OVI's (Organic Volatile Impurities)

For the separation of solvents, different columns are available. Frequently cyanopropylphenyl-methyl-silicone are used but methyl-phenyl-silicones or polyethylene glycol are useful if a different polarity is needed for separation of co-eluting solvents.
Headspace injection requires an amount of sample which sometimes is not available. Direct injection of a solution of the peptide in a suitable solvent such as e.g. DSMO requires as little as 1mg of the homogeneous sample. Limits of quantitation are in the lower ppm range. Understandably, the injection consumables (liner, filter) need to be replaced after several injections.

Determination of counter ions

After esterification, methyl acetate and trifluoroethyl acetate are separated using 5µm methylsilicone column. Pentafluoropropionic acid is used as internal standard. The sample is allowed to react with methyl alcohol/BF3, whereby the carboxyl groups including the trifluoroacetyl ions and acetyl ions are converted to their methyl esters. After careful extraction with hexane the esters are determined by capillary gas chromatography.



Advantage:

- Even strongly adsorbed ions or covalently linked acetate and trifluoroacetates can be determined.
- Validation of the method shows that the standard deviation of the determination of both ions is +/- 7% over the concentration range of 0.1 to 10%.

Determination of moisture

The concentration of water in solids is an important parameter in many fields - e.g. in the determination of the content of a peptidic sample, whereby the results from the amino acid analysis are verified by the counter-ion determination, the content of residual organic solvents and the water content, the sum of which, together with the peptidic content, should amount to 100%. Both the synthesis strategy and the final dosage are dependent upon these values.

In contrast to the indirect methods of determination of water whereby products of reaction with water are quantified, the chromatographic method measures water directly. After thermal desorption at 140°C, water is separated from other volatile components and detected quantitatively. Quantitation is done by the external standard method.
Advantage:

- The determination of water is not interfered with by other hydroxyl-containing components.
- Other volatile components do not falsify results.
- The amount of sample required is approx. 1 mg
- The relative standard deviation of the analysis is 5%.
- The limit of quantitation is approx. 0,05 %.

References:

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