Validation of a GC-MS Method for Determination of the Optical Purity of Peptides.

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Abstract:

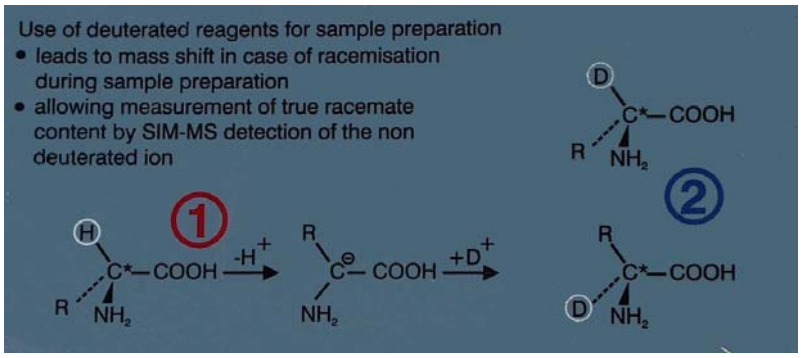
Measurement of the racemate content is an important aspect of the determination of the purity of peptidic products. One of the most accurate and sensitive methods for the analysis of the optical purity of free amino acids involves derivatization and gas chromatographic separation of the enantiomers using a chiral stationary phase. A technique for the unambiguous 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].

In this poster, validation of the method described in [3] for all proteinogenic and several non-proteinogenic amino acids is presented. 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.

Method:

Racemization during acid hydrolysis involves protonation and deprotonation 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:

$$\text{Original racemate content} = \frac{100 * F_{D(I)}}{F_{D(I)} + F_{L(I)}}$$

Two 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.
Interference from neighboring ions $(I+1)^+$ and $(I-1)^+$ could be taken into account using multiple ion detection using a series of equations [2].

Sample preparation:
100nmol of a mixture of amino acids is hydrolyzed in 300µl 6N DCl in D_2O for 24h at 110°C. After removal of excess of reagent by a steam of nitrogen, the sample is esterified with 350µl 2N deuterochloric acid-methyl alcohol for 15 min. at 110°C. After cooling to about 50°C the vial is opened and the reagent is evaporate with a gentle steam of nitrogen at moderate temperature. The residue is dissolved in 250µl trifluoroacetic anhydrid/trifluoroacetate and the vial is tightly closed and heated for 10min. to 130°C. The residue is dissolved in 150µl toluene and injected.

Gas Chromatography:
The N(O,S)-triflouroacetyl amino acid esters are separated on a deactivated glass capillary coated with Chirasil-Val or Cyclodextrin (for N-methyl-amino acids).

Columns:	Chirasil-Val, dimensions 20m*0.28 mm
	Cyclodextrin , dimension 20m*0.25 mm
Carrier gas:	Hydrogen
Flows:	Carrier gas 1,5ml/min, Split: 35ml/min,
	Purge 3ml/min
Temperatures:	Injector: 190°C; Detector 250°C;
	Oven 65°C isotherm for 2 min, 3°C/min to 80°C, 5°C/min 185°C.

The peaks are identified by their retention times.

Apparatus:

Gas chromatograph-mass spectrometer:	Varian SATURN 2000 / HP 5973
Integrator:	Saturn 2000 Software / HP Chem
Vials, closures and heat block:	C.A.T. H250, H500 and H103

Results:

Characteristic of Precision (repeatability):

The amino acids will be spiked with an aliquot of the optical antipode in order to be able to determine the standard deviation at a concentration level >LOQ. 6 aliquots are analyzed separately including the whole sample preparation.
The confidence interval of the mean value (a=0.05, 95% probability) is determined using the range of dispersion calculated with the Student-t-factor t(95%, a=0.05).

	Ala	Val	Ile	Ser	Cys	Met	Tyr	Orn	Trp	Thr	Pro	Leu	Asp	Glu	Phe	p-CPh	Lys	Arg
	0,272	0,188	0,223	0,689	0,173	0,397	0,298	0,171	1,284	0,078	0,138	0,144	0,177	0,185	0,109	0,162	0,165	0,112
	0,181	0,182	0,273	0,647	0,150	0,414	0,260	0,168	1,068	0,065	0,095	0,124	0,144	0,172	0,084	0,129	0,153	0,143
	0,218	0,162	0,253	0,636	0,159	0,402	0,290	0,187	0,992	0,067	0,107	0,124	0,154	0,221	0,107	0,147	0,164	0,155
	0,214	0,153	0,251	0,643	0,138	0,418	0,246	0,170	0,969	0,070	0,101	0,107	0,140	0,229	0,093	0,139	0,167	0,168
	0,222	0,204	0,295	0,670	0,175	0,416	0,297	0,177	1,167	0,057	0,106	0,114	0,140	0,155	0,081	0,121	0,160	0,181
	0,241	0,198	0,239	0,680	0,150	0,405	0,288	0,239	1,080	0,060	0,105	0,131	0,152	0,217	0,094	0,137	0,160	0,126
Mean value	0,225	0,181	0,256	0,661	0,158	0,409	0,280	0,185	1,093	0,066	0,109	0,124	0,151	0,197	0,095	0,139	0,162	0,148
Standard deviation	**0,03**	**0,02**	**0,025**	**0,022**	**0,014**	**0,009**	**0,022**	**0,027**	**0,117**	**0,007**	**0,015**	**0,013**	**0,014**	**0,03**	**0,012**	**0,014**	**0,005**	**0,026**
relative Std.	13,47	11,07	9,928	3,295	9,164	2,086	7,719	14,66	10,69	11,29	13,83	10,44	9,246	15,32	12,15	10,27	3,102	17,53
Confidence interval 95%	0,032	0,021	0,027	0,023	0,015	0,009	0,023	0,029	0,123	0,008	0,016	0,014	0,015	0,032	0,012	0,015	0,005	0,027
Confidence interval of mean value from	0,193	0,160	0,229	0,638	0,142	0,400	0,257	0,157	0,971	0,058	0,093	0,110	0,136	0,165	0,083	0,124	0,156	0,120
to	0,256	0,202	0,282	0,684	0,173	0,418	0,303	0,214	1,216	0,074	0,124	0,138	0,166	0,228	0,107	0,154	0,167	0,175

The precision shown that standard deviation of determination of the optical purity is <0.1% for all amino acids with the exception of Trp which is 0.12%

Intermediate precision:

Another 3 aliquots of the spiked mixture is determined on a different day.

The intermediate precision is evaluated statistically:

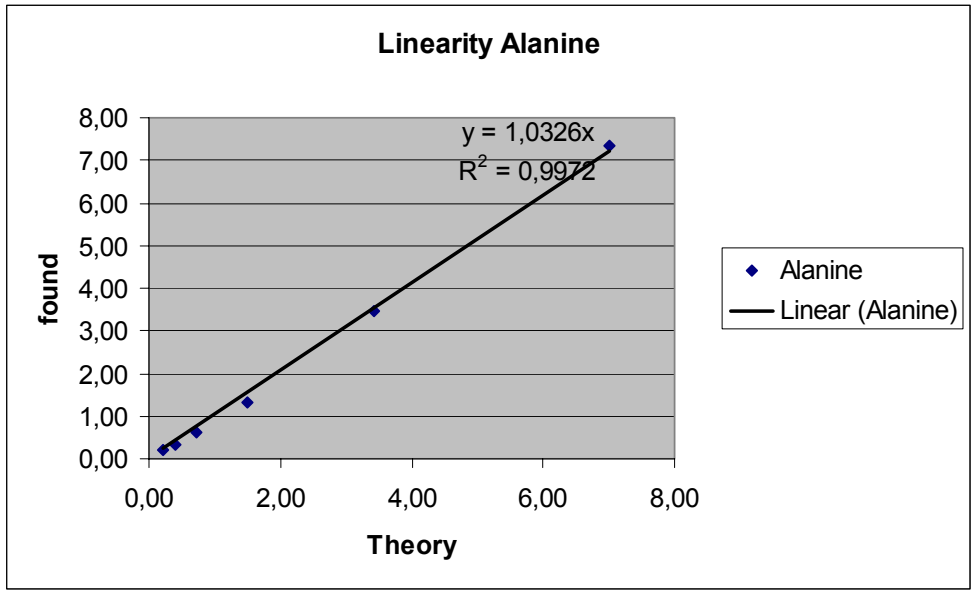
T-test and F-test of 3 runs against the 6 runs of the Precision. F-test returns the probability value of the variances being "equal". A value >0,05 indicates that the variances of the two sets of results are not different. T-test (tails=2, type=2) returns the probability value of the mean values being "equal". A value >0,05 indicates that the mean values of the two sets of results are not different.

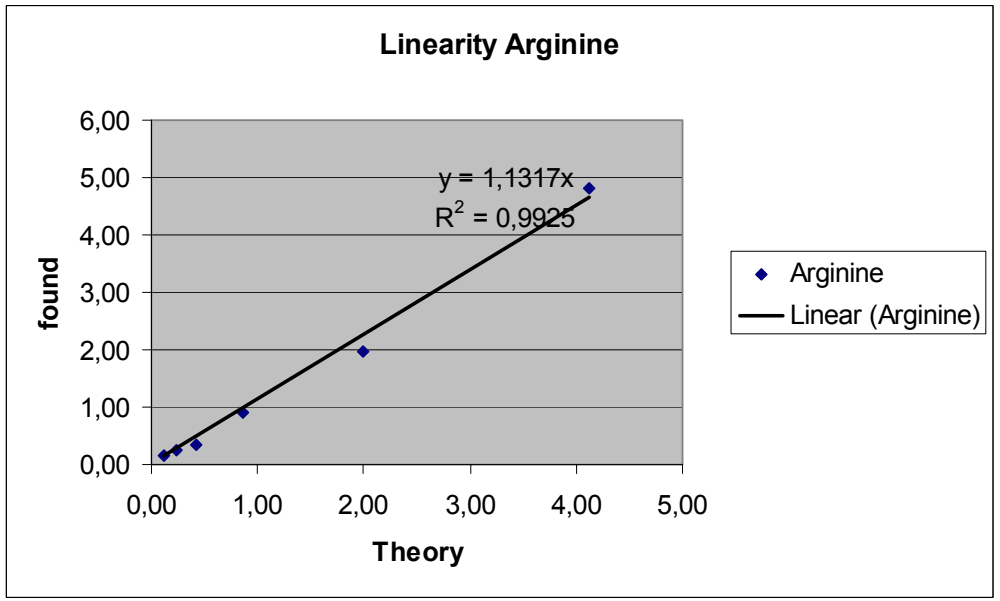
	0,254	0,208	0,279	0,625	0,197	0,390	0,316	0,202	0,942	0,062	0,128	0,118	0,130	0,177	0,100	0,144	0,159	0,135
	0,265	0,269	0,255	0,672	0,209	0,410	0,319	0,208	0,783	0,101	0,073	0,143	0,162	0,263	0,114	0,136	0,153	0,179
	0,214	0,179	0,237	0,594	0,145	0,403	0,309	0,185	1,062	0,097	0..075	0,136	0,147	0,336	0,109	0,115	0,162	0,157
F-Test	0,99	0,12	0,91	0,25	0,11	0,65	0,11	0,34	0,64	0,05	0,10	0,87	0,70	0,07	0,60	0,80	0,97	0,94
T-Test	0,37	0,12	0,94	0,17	0,13	0,27	0,03	0,47	0,10	0,06	0,65	0,39	0,65	0,12	0,12	0,49	0,35	0,60

Characteristic of Linearity:

The linearity is determined by adding the optical antipodes in 6 different levels of concentration.
The linearity is determined by the correlation coefficient and slope of the regression line. The correlation coefficient is >0.99. The plot of the signal versus the concentration of the optical antipode is linear by visual inspection.

	Ala	Val	Ile	Ser	Cys	Met	Tyr	Orn	Trp	Thr	Pro	Leu	Asp	Glu	Phe	p-CPH	Lys	Arg
correlation coefficient	0,998	0,995	0,995	0,996	0,991	0,998	0,997	0,998	0,997	0,993	0,992	0,993	0,994	0,993	0,991	0,994	0,996	0,993
slope	1,10	1,12	1,13	0,941	0,982	0,997	0,981	0,95	1,05	1,18	1,08	1,07	1,09	1,04	1,08	1,05	1,06	1,13





Characteristic of Accuracy:

The accuracy will be calculated from the data of linearity.
The percent recovery is determined from the data of linearity. The mean value of recovery and the standard deviation is calculated.
The recovery should be 85%-115%.

	Ala	Val	Ile	Ser	Cys	Met	Tyr	Orn	Trp	Thr	Pro	Leu	Asp	Glu	Phe	p-CPh	Lys	Arg
	114,3	100,2	102,8	124,7	85,5	126,8	125,9	102,1	100,0	89,7	117,6	110,5	110,4	109,7	113,4	110,5	94,8	119,4
	98,1	99,8	113,9	122,0	86,3	102,9	127,1	102,1	115,3	83,8	92,2	90,2	114,1	116,4	86,2	94,2	112,0	106,3
	90,7	91,3	90,0	130,9	79,6	123,8	108,8	89,7	87,2	87,1	84,4	88,8	109,8	102,8	83,7	83,0	84,9	85,2
	99,0	95,1	96,3	102,9	88,2	103,7	99,5	98,0	106,9	89,9	89,0	85,7	91,3	88,1	90,3	90,4	95,4	104,9
	106,5	101,9	103,9	90,6	85,7	94,1	91,0	87,9	112,8	110,0	96,5	97,0	98,5	91,5	95,5	94,5	98,1	99,2
	111,5	115,3	116,7	94,1	102,2	100,7	99,5	96,9	102,6	121,5	112,2	110,5	112,1	107,3	112,3	108,0	108,7	117,1
Mean value	103,3	100,6	104,0	110,9	87,9	108,7	108,6	96,1	104,1	97,0	98,7	97,1	106,0	102,6	96,9	96,8	99,0	105,4
Standard deviation	**9,0**	**8,2**	**10,7**	**17,2**	**7,6**	**13,4**	**15,0**	**6,1**	**10,1**	**15,1**	**13,3**	**11,0**	**9,1**	**10,9**	**13,0**	**10,6**	**9,9**	**12,5**

Characteristic of LOQ and LOD:

The limit of quantitation is determined as a signal to noise ratio of 10:1 from a sample of known low concentration. The limit of detection is determined as a signal to noise ratio of 3:1 from a sample of known low concentration.

	Ala	Val	Ile	Ser	Cys	Met	Tyr	Orn	Trp	Thr	Pro	Leu	Asp	Glu	Phe	p-CPH	Lys	Arg
LOQ [%]	0.1%	0.1%	0.2%	0.1%	0.2%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%	0.1%

Characteristic of Selectivity:

Chromatograms of each derivative will show the selectivity of the method. Chromatograms are obtained from determination of the precision.

Calculations:

Calculation of the optical purity:

$$\%\,D = \frac{Area_D}{Area_D + Area_L} * 100$$

Mean value

$$\overline{x}_i = \frac{1}{n_{(i)}} \sum_{k=1}^{n(i)} x_{ik}$$

Standard deviation

$$s_i = \pm \sqrt{\frac{1}{n(i)-1} \sum_{k=1}^{n(i)} (x_{ik} - \bar{x}_i)^2}$$

Relative Standard deviation

$$CV_i = \frac{s_i}{\overline{X}_i} * 100\ \%$$

Confidence interval

$$\overline{X}_i \pm \frac{s_i * t_{(95\%,\ f)}}{\sqrt{n_i}}$$

$t_{95\%,\ f}$ = Student t-Faktor ($f = n_{(i)} - 1$) (s.Tab.)

Correlation coefficient

$$r = \frac{CV_{(X,Y)}}{\sigma_x * \sigma_y}$$

where

$$\sigma_i = \sqrt{\frac{1}{n(i)} \sum_{k=1}^{n(i)} (x_{ik} - \bar{x}_i)^2}$$

$$CV_{X,Y} = \frac{1}{n} \sum_{i=1}^{n} (x_i - \bar{x}) * (y_i - \bar{y})$$

$R^2=r^2$

Tab.: Student t-Faktor

f	P=95 %; α=0.05	P=99%; α=0.01	P=99.9%; α=0.001
1	12,706	63,657	636,619
2	4,303	9,925	31,598
3	3,182	5,841	12,924
4	2,776	4,604	8,610
5	2,571	4,032	6,869
6	2,447	3,707	5,959
7	2,365	3,499	5,408
8	2,306	3,355	5,041
9	2,262	3,250	4,781
10	2,228	3,169	4,587
11	2,201	3,106	4,437
12	2,179	3,055	4,318

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