Amino Acid Analysis Using DABS-CL

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Key Words

Reversed-phase HPLC
Pre-column derivatisation
DABS
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Summary

Pre-column derivatization followed by reversed-phase HPLC has become widely used as a sensitive and speedy method of amino acid analysis.

The technique has been improved by the development of simple and reproducible strategies for derivatization of the amino acids with DABS and for the HPLC analysis. Results obtained indicate that the method has a high sensitivity is well and suited to generate precise amino acid composition information from peptides and proteins.

Introduction

Many different methods have been proposed for amino acid analysis using HPLC with pre-column derivatisation. The most common reagents used are PITC [1] (phenylisothiocyanate) and OPA [2] (orthophthalic dialdehyde). Recently FMOC [3, 4] (fluorenyl-ethyl-chloroformate) and a combined derivatisation method of FMOC and OPA has created interest.

Of the different methods the use of DABS-CL (dimethylaminoazobenzenesulphonyl chloride), proposed by Chang, represents a significant improvement in the field of amino acid analysis because of its high sensitivity [5, 6, 7]. Although this method has been known for a number of years, it could not be brought into general use, mainly due to difficulties in achieving complete separation of all the compounds of interest in one run.

This work describes a separation of all amino acids occurring in hydrolysate samples by the use of a reversed-phase colum and with light absorption detection in the visible region.

Material and Methods

Chemicals

Dabsylchloride (4-dimethylaminoazobenzene – 4′-sulfonylchloride) was purchased from Pierce Chemicals (Rockford, Il., USA). PIPES (1,4-piperazine-diethanesulfonic acid) and citric acid from Sigma (Munich, FRG).

Ultrasphere ODS 4,6 × 150mm with a particle diameter of 5μm was used as reversed-phase support.

Stock Solutions

1. 4mM Dabsyl-chloride in acetonitrile (1.3mg/ml): Approximately 20mg Dabsyl-chloride in a 20ml vial, diluted with acetonitrile to a final concentration of 1.3mg/ml and stirred for 5 minutes at ambient temperature. While mixing continously, 1ml aliquots were dispensed into small screwcap vieals and immediately frozen (−20°C).
2. 100mM $NaHCO_3$, pH 8.3, stored in refrigerator (4°C).
3. Reaction diluent: 100ml of ethyl alcohol was slowly added to 100ml of 50mM Na_2HPO_4, pH 7.0. The solution was tightly sealed and stored at room temperature.
4. Sodium citrate (100mM citric acid): 12g (300mmol) NaOH was dissolved in 950ml water. 19.2g (100mmol) citric acid was added and, when solubilized, adjusted to 1000ml with water; pH 6.4.
5. Sodium PIPES (100mM PIPES sulfonic acid): 5.44g (136mmol) NaOH was dissolved in 950ml water. 30.24g (100mmol) PIPES sulfonic acid was added and when solubilized adjusted to 1000ml with water; pH 6.4.
6. 4% DMF in acetonitrile.

DABS Reaction Mixtures

For the analysis from liquid samples: to 25μl amino acid solution, 25μl water or internal standard, 50μl 100mM $NaHCO_3$, pH 8.3, and 200μl 4mM DABS was added and thoroughly vortex mixted. The solution was incubated at 70–72°C for 12 minutes, cooled for 5 minutes and 700μl of reaction diluent added. The injection volume was 20μl.

To an appropriate amount of dry samples, 50μl water or internal standard solution was added and the procedure described for liquid samples was then followed.

For microhydrolysates the derivatisation reaction can be performed with one fifth of the above scale.

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Equipment

The instrumentation consisted of a gradient HPLC System Gold with a Module 166 detector using a tungsten lamp for detection at the fixed wavelength of 436nm.

Peak area integration was achieved with the System Gold software. The chromatographic conditions are shown in Table I.

Table I. Composition of Eluent A and B for a 150mm Ultrasphere ODS column. Gradient program for a amino acid standard mixture (hydrolysate)

Mobile Phase for 4.6 X 150mm Ultraphsere ODS*				
"A" component: (pH 6.5 ± 0.05):				
. 200mL sodium citrate				
. 40mL PIPES				
. 720mL water				
. 40mL DMF				
"B" component:				
To 300mL of "A" component, add slowly with constant mixing 700mL 4% DMF in CH_3CN.				
		Gradient		
Time	Rate	%A	%B	Dur. min
Init.	1.40mL/min	71	29	
0.00		49	51	12
12.00		14	86	5
17.00		0	100	1.00
27.00		71	29	0.25

Results

Complete separation of all DABS-amino acids is shown in Figure 1. DABS-Cl reacts with all amino acids including proline, hydroxyproline, cysteine and tryptophane. Hydrolized excess reagent DABS-CL appears in the chromatogram but does not interfere with amino acids. Norleucine was used as internal standard.

Statistical evaluation of the derivatization chemistry and system reproducibility of DABS-amino acid analysis was performed running six replicate mixtures. Table II shows the results for absolute peak areas and absolute retention times (19.5 pico mole amino acid standard mixture in 30µl injection volume).

An outstanding feature of DABS-amino acids is their stability over a long period. Table III compares results from 10 minutes, 1 week, 2 weeks and 4 weeks changes in concentrations relative to leucine.

Conclusion

The DABS-Cl analysis is potentially more sensitive than the conventional amino acid analyzer (Spackman, Moore & Stein [8]). Using absorption detection at 436nm, 5pm of amino acid is reasonable for routine analysis. The detectable quantities claimed for the fluorescence detection method, where sensitivities up to femto (10^{-15}) mole ranges are quoted, cannot be reached by this method. However, often no attention is paid to the fact that this claim

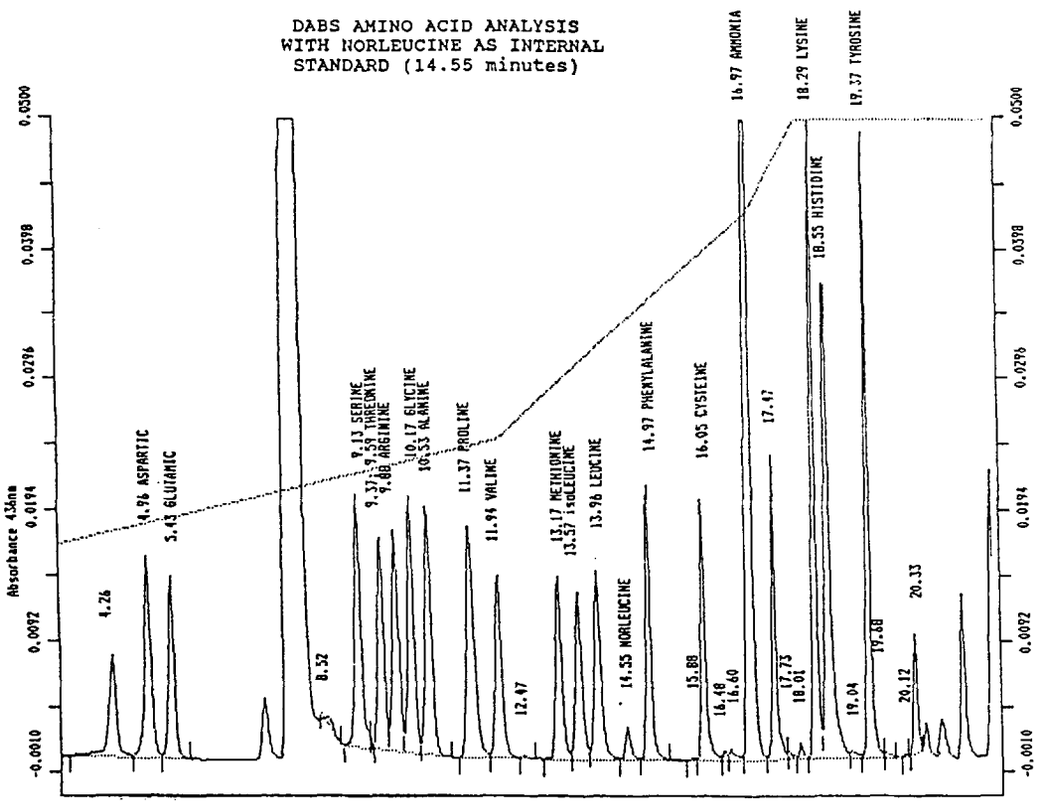


Figure 1

DABS amino acid analysis with Norleucine as internal standard (14.55 minutes). Chromatogram of a standard amino acid run, conditions are given in Table I.

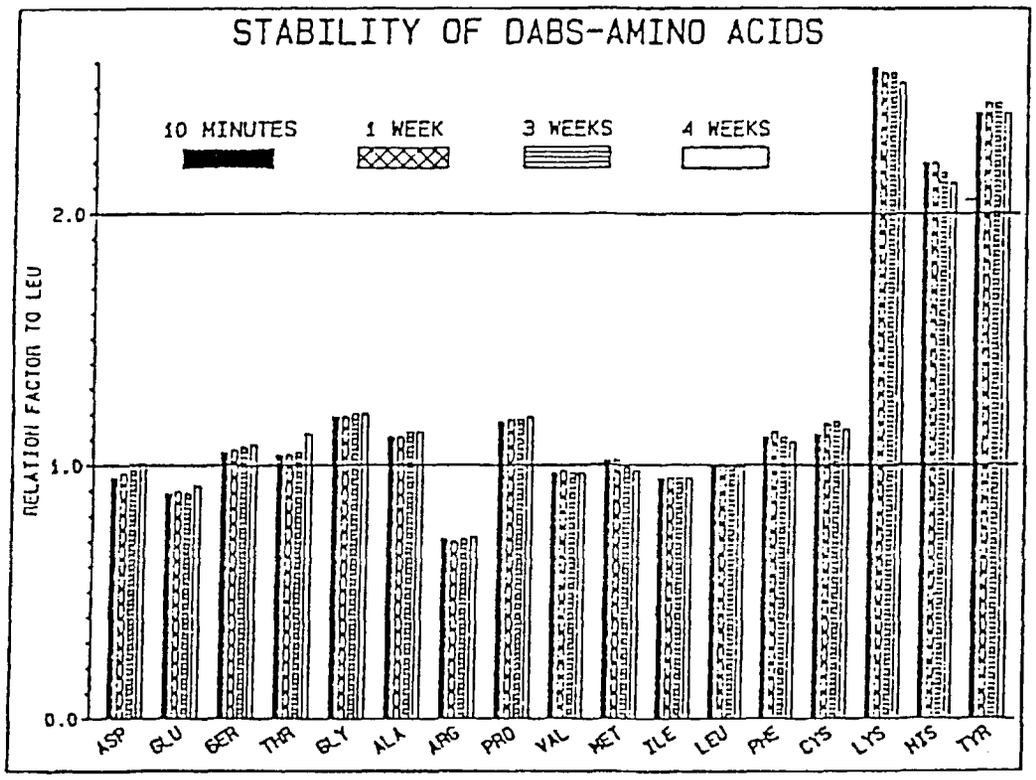
Table II. Statistical evaluation of DABS-amino acid analysis of six replicate runs.
- Upper part: Results for absolute peak areas
- Lower part: Results for absolute retention times

System gold DABS amino acid precision data reverse phase 19,5 picomole im 20µL 136mM									
					Absolute peak areas				
Sample	ASP	GLU	SER	THR	ARG	GLY	ALA	PRO	VAL
19.5A	0.338	0.263	0.331	0.298	0.289	0.367	0.374	0.419	0.291
19.5B	0.334	0.253	0.320	0.287	0.281	0.356	0.361	0.405	0.272
19.5C	0.330	0.255	0.314	0.291	0.283	0.358	0.370	0.411	0.281
19.5D	0.342	0.264	0.324	0.300	0.290	0.361	0.373	0.402	0.281
19.5E	0.334	0.256	0.315	0.291	0.284	0.359	0.322	0.413	0.277
19.5F	0.321	0.254	0.313	0.290	0.282	0.360	0.362	0.409	0.284
Mean	0.333	0.258	0.320	0.293	0.285	0.360	0.369	0.410	0.281
STD DEV	0.007	0.004	0.006	0.005	0.003	0.003	0.005	0.005	0.006
REL STD DEV	1.98%	1.69%	2.00%	1.57%	1.21%	0.95%	1.24%	1.34%	2.09%
					Absolute retention times				
Sample	ASP	GLU	SER	THR	ARG	GLY	ALA	PRO	VAL
19.5A	1.879	5.331	9.016	9.476	9.745	10.050	10.420	11.296	11.854
19.5B	4.893	5.350	9.035	9.494	9.766	10.071	10.436	11.308	11.864
19.5C	4.885	5.340	9.032	9.494	9.762	10.067	10.432	11.303	11.857
19.5D	4.881	5.336	9.031	9.491	9.761	10.065	10.430	11.301	11.856
19.5E	4.872	5.327	9.018	9.481	9.749	10.051	10.481	11.293	11.847
19.5F	4.862	5.315	9.008	9.473	9.473	9.741	10.416	11.296	11.055
Mean	4.879	5.331	9.023	9.485	9.754	10.436	10.436	11.300	11.856
STD DEV	0.010	0.011	0.010	0.009	0.009	0.010	0.021	0.005	0.005
REL STD DEV	0.20%	0.20%	0.11%	0.09%	0.10%	0.10%	0.20%	0.04%	0.04%
					Absolute peak areas				
Sample	MET	ILE	LEU	PHE	CYS	LYS	HIS	TYR	MTYR
19.5A	0.299	0.282	0.297	0.320	0.265	0.572	0.605	0.499	0.723
19.5B	0.280	0.263	0.277	0.303	0.262	0.553	0.604	0.482	0.704
19.5C	0.293	0.272	0.288	0.315	0.264	0.565	0.597	0.493	0.723
19.5D	0.294	0.271	0.288	0.314	0.275	0.569	0.605	0.505	0.742
19.5E	0.287	0.266	0.280	0.310	0.264	0.560	0.595	0.474	0.209
19.5F	0.288	0.275	0.287	0.313	0.260	0.574	0.579	0.494	0.732
Mean	0.290	0.272	0.286	0.313	0.265	0.566	0.598	0.491	0.722
STD DEV	0.006	0.006	0.006	0.005	0.005	0.007	0.009	0.010	0.013
REL STD DEV	2.08%	2.26%	2.24%	1.66%	1.80%	1.28%	1.53%	2.11%	1.78%
					Absolute retention times				
Sample	MET	ILE	LEU	PHE	CYS	LYS	HIS	TYR	MTYR
19.5A	13.068	13.504	13.881	14.910	16.022	18.260	18.538	19.345	19.494
19.5B	13.080	13.514	13.889	14.917	16.024	18.266	18.544	19.352	19.501
19.5C	13.065	13.502	14.878	14.910	16.020	18.263	18.542	19.349	19.499
19.5D	13.070	13.508	13.884	14.914	16.021	18.263	18.540	19.348	19.499
19.5E	13.058	13.499	13.875	14.905	16.018	18.260	18.538	19.345	19.495
19.5F	13.068	13.510	13.886	14.918	16.033	18.265	18.546	19.351	19.499
MEAN	13.068	13.506	13.882	14.912	16.023	18.263	18.541	19.348	19.498
STD DEV	0.007	0.005	0.005	0.004	0.005	0.002	0.003	0.003	0.002
REL STD DEV	0.05%	0.04%	0.03%	0.03%	0.03%	0.01%	0.02%	0.01%	0.01%

describes the theoretical performance of the fluorescence detectors with aliquotes of a higher quantity of standard mixture being injected directly. The problems in handling such small quatities are due to sample preparation (hydrolysis) and contamination. Using real samples in routine analysis, sensitivities in the lower pico (10^{-12}) mole range will certainly be sufficient.

As described in the experimental section above derivatisation requires heating of the samples at 70°C for 12 minutes. For this step a simple heater block was used, with all samples incubated simultaneously. Compared to the method of evaporation or extraction this step is much more convenient. Due to the stability of the derivatives the method can be fully automated, after this step, by using an

Table III. Comparison of the stability of DABS-amino acids from 10 minutes to 4 weeks. Absolute areas relative to leucine



autosampler. Even using the autosampler at full capacity no distortion of the results will occur since the amino acid derivatives are very stable.

By the use of our procedure primary and secondary amino acids can be detected, which is an important advantage in comparison with the OPA-method [2]. It requires simple instrumentation and the running costs are low in comparison with the Ninhydrin-method. In general it is a robust, sensitive and easy-to-use method for hydrolysate amino acid analysis with a potential also for physiological applications up to about 35 amines.

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