A Validated Analysis of Underivatized Amino Acids Using HPLC with Evaporative Light Scattering Detection

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The analysis of amino acids is typically performed via HPLC by formation of a derivative (such as OPA or FMOC) followed by separation and detection via absorbance or fluorescence since the most common amino acids do not contain a chromophoric group. While these techniques provide satisfactory resolution and sensitivity, the derivatization step adds an extra level of complexity, cost, and time for the analysis. In contrast, the use of evaporative light scattering detection is capable of monitoring all amino acids without the need for derivative formation, since it is a mass sensitive technique, rather than relying on the presence of a chromophoric group.

In this note, we describe the analysis of a series of amino acids using reverse phase separation with an evaporative light scattering detection to provide a sensitive, reproducible, and robust analytical procedure.

Experimental Conditions

A certified commercial sample of amino acids (Thr, Lys, Val, Met, Ile, Leu, Phe, Trp) was separated using a Merck Purosphere RP-18 (125 × 4 mm 5 µm) column using a gradient of A: 0.1% heptafluorobutyric acid, B: acetonitrile (0–2 min: 100% A. 2–17 min 100 % A to 70 % A, linear gradient, flow 1.0 mL/min. Injection volume = 20 µL (sample concentration 5–500 µg/mL).

Detection was performed with a SEDEX Low-Temperature Evaporative Light Scattering Detector (Model 55) with the evaporation temperature set to 60 °C, the gain to 9 and a pressure of 2.3 bar.

Results

A sample chromatogram is presented in Figure 1; it is clear that the amino acids are well separated and readily detected by the ELSD detector.

When ELSD is employed, a log/log relationship is used to determine the concentration of the compound of interest (Eq. 1):

$$\log (\text{Area}) = a \log (\text{concentration}) + b \qquad [1]$$

where a and b are constants.

Analysis of a series of six standards samples provided a limit of detection (LOD) of 1–2 µg/mL and a limit of quantification (LOQ) of 5–10 µg/mL for the various amino acids, with r^2 values between 0.9994 and 1.0000, clearly indicating that a reliable quantitation was obtained.

The analysis was run on a series of days as shown in Table I. These experiments demonstrate that the analysis provides concentrations that correspond to the certified value. Each analysis was done three times and the results were averaged.

The long term performance of the assay is shown in Table II, which presents the retention factors that were obtained when the assay was first performed and the retention factors obtained 26 months later using a different column, different operator, and different analytical system. This data clearly indicates that this assay is reproducible over a long period of time and with different chromatographic systems.

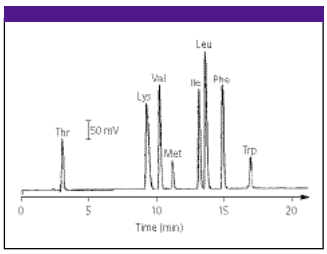

Figure 1: Separation of amino acids stationary phase: Merck Purosphere RP-18 (125 × 4 mm 5 µm) column Mobile Phase: Gradient A: 0.1% heptafluorobutyric acid, B: acetonitrile (0–2 min: 100% A. 2–17 min 100 % A to 70 % A, linear gradient, flow 1.0 mL/min. Injection volume = 20 µL (sample concentration 5–500 µg/mL). Detection was performed with a SEDEX Low-Temperature Evaporative Light Scattering Detector (Model 55) with the evaporation temperature set to 60 °C, the gain to 9 and a pressure of 2.3 bar.

The robustness of the method was determined by varying the pH and the ion pairing concentration as shown in Table III (the ion pairing concentration cannot be buffered since perfluoroacids form nonvolatile salts with bases, thereby decreasing the sensitivity of detection).

Conclusions

Quantitation of underivatized amino acids by reverse phase chromatography with evaporative light scattering detection provides the analyst with a sensitive analytical procedure that is simpler and more straightforward than the use of derivatized samples.

Evaporative light scattering detection is capable of monitoring essentially all compounds in the HPLC sample and does not require a chromophoric group. The quantitative protocol for amino acids that is described herein is easy to perform and is reproducible, robust, and can be readily validated.

Table I: Analytical data

Amino Acid	Certificate of Analysis	Day 1	Day 4	Day 7	% RSD
Thr	10.85	10.76	11.39	10.88	3.04
Lys	17.15	17.05	18.25	17.38	3.53
Val	15.75	15.8	16.68	16.09	2.77
Met	6.65	6.74	7.04	6.85	2.21
Ile	14.14	14.07	14.77	14.22	2.57
Leu	18.76	18.94	19.58	19.3	1.66
Phe	12.54	12.08	12.8	12.21	3.10
Trp	3.47	3.5	3.54	3.65	2.18

Table II: Retention factors for amino acids

Amino Acid	Retention Factors on the First Day	Retention Factors 26 Months Later
Thr	2.02	2.03
Lys	8.34	8.26
Val	9.32	9.01
Met	10.24	9.83
Ile	12.27	11.74
Leu	12.77	12.24
Phe	14.03	13.36
Trp	16.09	15.33

Table III: Robustness of the assay

HFBA Conc.	6.6 mM	7.1 mM	7.6 mM	8.1 mM	8.6 mM	
pH	**2.18**	**2.11**	**2.07**	**2.06**	**2.02**	**% RSD**
Thr	2.76	2.87	3.03	3.06	3.11	4.92
Lys	8.28	8.82	9.26	9.43	9.54	5.71
Val	9.62	9.82	10.01	10.06	10.1	2.02
Met	10.33	10.57	10.82	10.87	10.97	2.42
Ile	12.48	12.58	12.74	12.81	12.91	1.37
Leu	12.95	13.04	13.23	13.28	13.41	1.41
Phe	14.04	14.14	14.36	14.42	14.61	1.59
Trp	16.05	16.07	16.33	16.39	16.57	1.36

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