Waters
Lab Highlights
An Internal Communication of Applications and Techniques
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High Sensitivity Pico-Tag® Analysis

Using the Pico-Tag method, one to ten micrograms of protein is a typical sample size from which excellent amino acid compositions can be routinely obtained. But analysts are now using the method to study even smaller quantities of protein which requires very high sensitivity. ***The real issue is not the level of sensitivity for standards, but how much sample is required to obtain an accurate amino acid composition*** of a protein or peptide.

We will address this issue in two parts. This Lab Highlight will address the factors which limit the sensitivity and accuracy of chromatographic analysis on the equivalent of a submicrogram samples of peptides and proteins. To this end we will use "standards." The second part, "What are the contamination issues that limit compositional analysis?" will be addressed in a subsequent Lab Highlight. When both of these Lab Highlights are considered, the important question of "can the Pico-Tag method do the best job" can be answered with a resounding "yes." A more extensive discussion on the topic of high sensitivity analysis is found in reference 1.

Several factors might be responsible for limiting the sensitivity and accuracy of chromatographic analysis on real (submicrogram) samples of proteins and peptides: a) molar absorptivity; that is, how much response is produced by an injection of a given quantity of amino acid, b) the contribution of baseline noise and drift, c) degradation, either of the amino acids or their derivatives which might lead to extra peaks and, d) interfering peaks, either from the PITC reagent or its by-products.

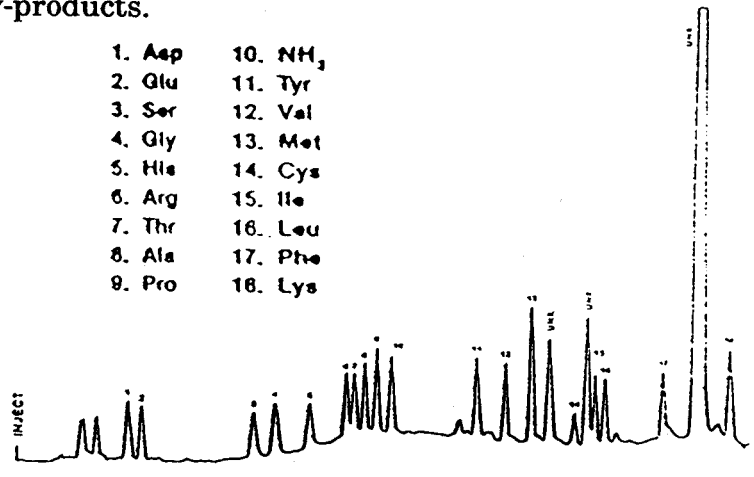

Figure 1. Separation of amino acid standards at 4 pmole level. Pico-Tag Column (3.9 X 15 mm). Time until the end of the Chromatogram is 12 minutes.

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Factors a) and b) are detection issues, and an examination of Figure 1 makes it clear that the molar absorptivity of the PTC derivatives is sufficient to produce peaks which are easily quantitated at less than 4 picomoles injected. For a 70,000 MW protein, the hydrolysis of 0.7 μg and injecting 10% would be approximately at the 4 picomole level. Also note that baseline noise and drift obtained when using the 440 are minimal. This will not be the case when using a 481 or a competitor spectrophotometer which lacks Taper-Cell® technology. These detectors will have more noise and baseline drift during the gradient.

Factors c) and d), concern additional peaks which may interfere with quantitation. It is apparent that the reagent/by-product peaks (labeled "UNK" in Figure 1) are now large enough to cause problems, specifically for isoleucine (peak number 15 in Figure 1). Also, if PTC-ASP converts to PTH-ASP due to lack of adequate removal of acid from the hydrolysis step, the PTH species will elute between proline and ammonia (peaks 9 and 10 in Figure 1).

To improve the situation one must improve resolution, so that the large reagent/by-product peaks are better separated from the peaks of interest. In order to accomplish this and still use the standard Pico-Tag eluents a 30 cm Pico-Tag Column for Free Amino Acid Analysis (Part #10950) should be used. Flow rate remains at 1 mL/min; gradient times are doubled and column temperature is increased to 46°C. The separation is shown in Figure 2.

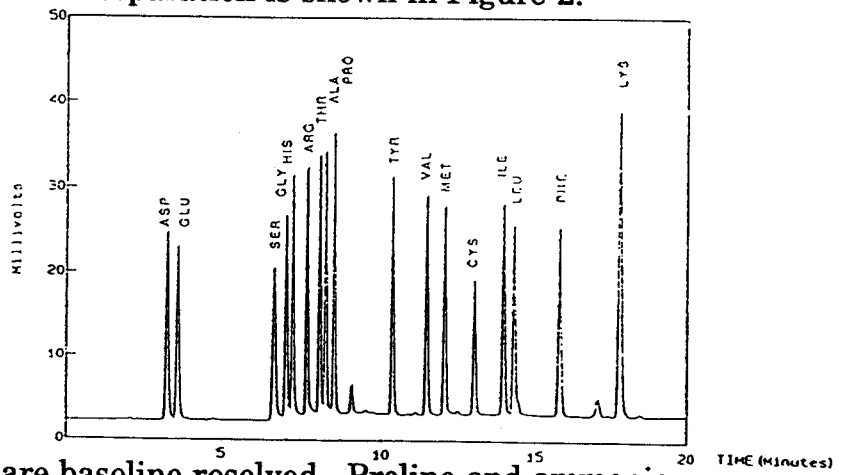

Figure 2. Separation of amino acid standards at 250 picomole level on 3.9 X 300 mm column at 46°C.

Note that all peaks are baseline resolved. Proline and ammonia are now very well resolved, and there is room for PTH-aspartic acid (formed by conversion from PTC-Asp). This improved resolution (Figure 2) will result in better compositional analyses at low levels, because it also reduces the interference by reagent/by-product peaks. Tips for minimizing the reagent/by-product peaks are contained in the soon to be released Pico-Tag Methods Book.(2) The conclusion is that for those researchers desiring to do compositional analysis at low levels, a 30 cm Pico-Tag column should be used.

References:

1) B. A. Bidlingmeyer, T. L. Tarvin, S. A. Cohen, "Amino Acid Analysis of Submicrogram Hydrolyzate Samples" in Methods in Protein Sequence Analysis 1986, Edited by Kenneth A. Walsh, The Humana Press, 1987.

2) Cohen, S. A., Meys, M., and Tarvin, T. L., in *The Pico-Tag Method - A Manual of Advanced Techniques for Amino Acid Analysis*, Millipore Corporation, in press.

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