AccQ•Tag SOLUTIONS

The Successful Use of UV Detection with the AccQ•Tag™ Method

Your laboratory needs a high-quality amino acid analysis method. You want to try the Waters AccQ•Tag method, but you currently have a Waters gradient HPLC with a UV detector, and you know that the AccQ•Tag method usually relies on fluorescence detection to provide selective and sensitive detection of the derivatized analytes. The optimized system includes a Waters 474 Scanning Fluorescence Detector routinely yielding detection limits from 50 - 300 fmol for amino acids in peptide and protein hydrolysates. Can you use your UV system? What compromises does UV detection incur? What is the expected performance of UV detection and are there any advantages? Why do some fluorescence detectors perform inadequately and seem no better than a UV detector? This application note provides answers to these questions.

Derivatization with Waters AccQ•Fluor™ Reagent

Waters AccQ•Fluor reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), was expressly designed with unique features for amino acid analysis that simplify derivatization, maximize derivatization yields and enhance detection selectivity and sensitivity using fluorescence detection[1]. The carbamate structure with the hydroxysuccinimide activating group is the key to rapid, quantitative derivatization of amine compounds. Other undesirable reactions are limited as the reagent is rapidly inactivated by hydrolysis which produces 6-aminoquinoline (AMQ). The quinoline tag on the analytes provides a highly sensitive, extremely stable label that can be stored at room temperature for at least a week.

The AccQ•Tag method relies on the difference in fluorescence properties between the derivatized amino acids and AMQ – the former having an emission maximum at 395 nm, the latter at 520 nm (excitation at 250 nm for both). This allows a large reagent excess to be used to drive the derivatization to completion, yet permits direct injection of the reaction mixture without reagent or side product removal. Only a small reagent peak is observed if fluorescence detection is employed with an emission setting at 395 nm.

The Basics of UV Detection

Since the absorption maxima for the AQC-derivatized amino acids are approximately 250 nm, fixed wavelength detectors operating at 254 nm as well as variable UV detectors are suitable for analysis. If a normal 50 pmol calibration standard is analyzed, approximately 10 nmol (a 200-fold excess) of AMQ is injected. Fluorescence detection results in an AMQ peak less than one-half the size of the weakest responding amino acid, but UV detection yields a peak 200-fold larger (Figure 1). You should note that the selective advantage of fluorescence detection is achieved only if a detector with two monochromators is used. Other detectors that use a long pass filter on the emission end yield as large a relative response for AMQ as UV detection. This enormous AMQ peak creates difficulties in quantitating Asp, the earliest eluting amino acid peak.

Sensitivity for most amino acids also suffers with UV detection. Detection limits are typically 0.5-1.0 pmol, 2-20 times worse than fluorescence detection. Good quantitation is also complicated when using Waters Eluent A concentrate for the AccQ•Tag

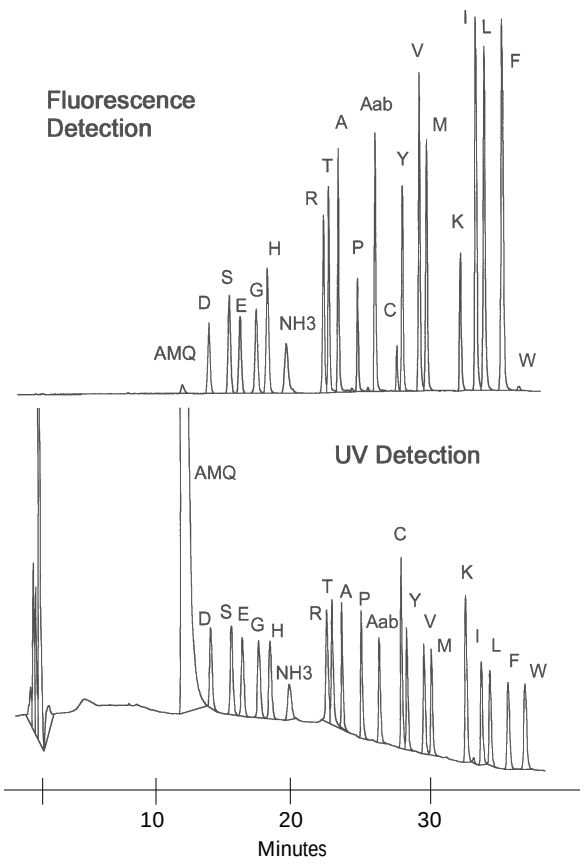

Figure 1 Comparison of hydrolysate standard mixture using UV (FS = 0.03 AU) and fluorescence (FS = 200mV) detection. Sample amount = 50 pmol. One letter amino acid codes are used. AMQ = 6-aminoquinoline, Aab = ∞-aminobutyric acid (internal standard).

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method. To ensure long shelf life and to prevent bacterial or fungal growth after reconstitution, Eluent A contains sodium azide as a preservative. While this has no effect on the separation and poses no problems for the fluorescence-based systems, the difference in UV absorption in the aqueous and organic eluents results in a significant baseline shift during analysis (Figure 1) on a UV system. This can cause quantitation difficulties that can be particularly severe at low pmol levels.

Practical Advice for Successful UV Operation

The first consideration is whether the resolution between Asp and AMQ is sufficient for your analyses. There are several simple ways to improve the separation without severely impairing the overall analysis. One of the most effective means is described in a recent publication[2] where the pH of Eluent A was reduced approximately 0.1 units. While this creates a larger gap between the critical peaks, it will also decrease resolution between Asp and Ser, as well as between Glu and Gly.

To reduce interference from background UV absorbance in the eluents, make Eluent A as described in the Operator's Manual, formulated without azide. Although the shelf life will be significantly less, the resulting baseline is flatter. High quality water and triethylamine are essential for best results. Careful titration of the eluent pH (± 0.05 units) is also required.

UV Detection – Yes for Trp!

The presence of two fluorescing groups on a single molecule may actually reduce fluorescence sensitivity due to a phenomenon know as internal quenching. This has been observed as a weak effect with Lys, but can be quite serious with Trp (Figure 1). In this instance, UV detection actually offers improved detection (1 pmol vs. 10 pmol limits). Samples with Trp, such as the analysis of free amino acids in an intravenous solution (see Figure 2), can be efficiently analyzed using two detectors in series. With the Waters 474 detector first in line, most amino acids are quantitated as usual, and the Trp is detected by the Waters 486 variable UV detector as the second.

Summary

Clearly, fluorescence is the recommended choice for detection, but UV detection can be attractive for trying the AccQ•Tag method on an existing HPLC system. If fluorescence detection is not an option, you now know the limitations and the options for optimizing the separation, allowing you to realize the advantages of the AccQ•Tag method.

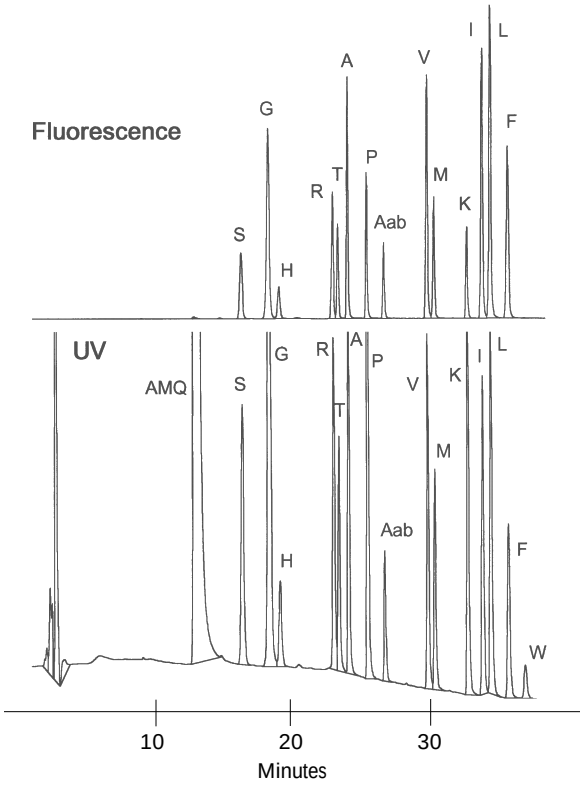

Figure 2 Comparison of intravenous solution analyses using UV (FS = 0.05AU) and fluorescence (FS = 600mV) detection. Sample preparation was (1) diluted the sample 100 fold with water (2) buffer an aliquot with the AccQ•Fluor derivatization buffer and (3) derivatize with the AccQ•Fluor reagent. Conditions and peak labels are given in Figure 1.

References

1. S.A. Cohen and D.P. Michaud, Anal. Biochem (1993), 211, 279-287.
2. Hong-Ji Liu, J. Chromatography (1994), 610, 59-66.
3. S.A. Cohen and K.M. De Antonis, J. Chromatography (1994), 661, 25-34.

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