ANNEX III

METHODS OF ANALYSIS TO CONTROL THE COMPOSITION OF FEED MATERIALS AND COMPOUND FEED

G.DETERMINATION OF TRYPTOPHAN

9. Observations

9.1. Following special chromatographic conditions may give better separation between tryptophan and α-methyl-tryptophan.

Isocratic elution followed by gradient column cleaning:

Liquid chromatographic column:	125 mm x 4 mm, C_{18}, 5 µm packing or equivalent		
Column temperature:	32 °C		
Mobile phase:	A: 0,01 mol/l KH_2PO_4/méthanol, 95+5 (V+V). B: methanol		
Gradient program:	0 min.	100 % A	0 % B
	15 min.	100 % A	0 % B
	17 min.	60 % A	40 % B
	19 min.	60 % A	40 % B
	21 min.	100 % A	0 % B
	33 min.	100 % A	0 % B
Flow rate:	1,2 ml/min.		
Total run time:	approx. 33 min.		

9.2. The chromatography will vary according to the type of HPLC and column packing material used. The chosen system must be capable of giving baseline separation between the tryptophan and the internal standard. Moreover it is important that degradation products are well separated from the tryptophan and the internal standard. Hydrolysates without internal standard shall be run in order to check the base line under the internal standard for impurities. It is important that the run time is sufficiently long for the elution of all the degradation products, otherwise late eluting peaks may interfere with subsequent chromatographic runs.

In the range of operation, the chromatographic system shall give linear response. The linear response shall be measured with a constant (the normal) concentration of the internal standard and varying concentrations of tryptophan. It is of importance that the size of both the tryptophan and internal standard peaks are within the linear range of the HPLC/fluorescence system. If either the tryptophan and/or the internal standard peak(s) is (are) too small or too high the analysis shall be repeated with another sample size and/or a changed final volume.

9.3. *Barium hydroxide*

With age barium hydroxide becomes more difficult to dissolve. This results in an unclear solution for the HPLC determination, which may produce low results for tryptophan.