buffers, react with phthalaldehyde and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this procedure may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution. Dissolve a suitable quantity of the substance to be examined in a 9 g/l solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions. Adjust the solution to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Reference solutions. Dissolve the reference substance for the protein to be determined in a 9 g/l solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/l solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/ml and 200 µg/ml. Adjust the solutions to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Blank solution. Use a 9 g/l solution of *sodium chloride R*.

Borate buffer solution. Dissolve 61.83 g of *boric acid R* in *distilled water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 ml with *distilled water R* and mix.

Phthalaldehyde stock solution. Dissolve 1.20 g of *phthalaldehyde R* in 1.5 ml of *methanol R*, add 100 ml of borate buffer solution and mix. Add 0.6 ml of a 300 g/l solution of *macrogol 23 lauryl ether R* and mix. Store at room temperature and use within 3 weeks.

Phthalaldehyde reagent. To 5 ml of phthalaldehyde stock solution add 15 µl of *2-mercaptoethanol R*. Prepare at least 30 min before use. Use within 24 h.

Procedure. Mix 10 µl of the test solution and of each of the reference solutions with 0.1 ml of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 ml of *0.5 M sodium hydroxide* and mix. Determine the fluorescent intensities (*2.2.21*) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

Calculations. The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis techniques destroy the test sample during the analysis but are not limited to protein presentation in an aqueous environment.

Procedure A. Proceed as prescribed for the determination of nitrogen by sulphuric acid digestion (*2.5.9*) or use commercial instrumentation for Kjeldahl nitrogen assay.

Procedure B. Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the substance to be examined. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal-conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2^*), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations. The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference substance.

01/2008:20534

2.5.34. ACETIC ACID IN SYNTHETIC PEPTIDES

Examine by liquid chromatography (*2.2.29*).

Test solution. Prepare as described in the monograph. The concentration of peptide in the solution may be adapted, depending on the expected amount of acetic acid in the sample.

Reference solution. Prepare a 0.10 g/l solution of *glacial acetic acid R* in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A.

The chromatographic procedure may be carried out using:

– a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),

– as mobile phase at a flow rate of 1.2 ml/min:

Mobile phase A. Dilute 0.7 ml of *phosphoric acid R* to 1000 ml with *water R*; adjust the pH to 3.0 with *strong sodium hydroxide solution R*,

Mobile phase B. *Methanol R2*,

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 5	95	5
5 - 10	95 → 50	5 → 50
10 - 20	50	50
20 - 22	50 → 95	50 → 5
22 - 30	95	5

– as detector a spectrophotometer set at 210 nm.

Inject 10 µl of the reference solution and 10 µl of the test solution. In the chromatograms obtained, the peak corresponding to acetic acid has a retention time of 3-4 min. The baseline presents a steep rise after the start of the linear gradient, which corresponds to the elution of the peptide from the column. Determine the content of acetic acid in the peptide.