Microbiological Assay of Folic Acid in Complete Feed and Premixes

Assay Parameter: Folic Acid

Product(s) to which applicable: Complete Feeds and Premixes

Assay range: Above 0.2 µg/kg

Principle: Turbidimetric

Coefficient of variation: 10%

Origin of Method (published or unpublished reference):

Roche Grenzach (No. 62 MV 060)

Analytical Methods for Vitamins and Carotenoids in Feeds, F. Hoffmann-La Roche & Co. AG, CH-4002, Basel, Switzerland

Reagents (and sources where applicable)

As far as possible, all reagents are of analytical grade.

1. Hydrochloric acid 0.1 mol/l

2. Sodium hydroxide 0.1 mol/l

3. Micro-Assay-Culture agar (Difco Laboratories, Detroit USA; No. 0319-02): 47 g are suspended in water and made up to 1 litre according to the instructions of the manufacturer.

4. Micro Inoculum Broth (MIB: Difco, no. 0320-02): 37 g are suspended in water and made up to 1 litre according to the instructions of the manufacturer.

5. Folic acid Assay Medium (test Nutrient mixture from Difco, no. 0318-15-0): **7.** 5 mg are suspended in water and filled up to 200 ml (single strength). Prepare a batch according to the instructions of the manufacturer.

6. Physiological saline solution: 8.5 g sodium chloride dissolved in water made up to 1 litre or Ringer's solution (Merck, Darmstadt, FRG; no. 15525: prepared according to the instructions of the manufacture). Sterilize for 30 mintes at 120° C and 1 bar.

7. Ammonia Solution 2%: 80 ml ammonia 25% are made up with distilled water to 1 litre.

8. Sodium ascorbate 10% aqueous solution

9. Sodium ascorbate 0.1 % aqueous solution

Apparatus (and sources where applicable)

1. Food process (e. g. Moulinex, France) or grinder (e. g. coffee grinder MX 32/MXK; Braun AG. Frankfurt/M, FRG)

2. Autoclave (e. g. Fedegari S. P. A., Albuzzano [PV], Italy).

3. Centrifuge (e. g. laboratory centrifuge 4000 rpm, Heraeus-Christ GmbH, Osterode, FRG; with 40-ml centrifuge tubes with ground stoppers).

4. pH-meter with glass electrode

5. Steamer or small autoclave (e. g. EGRO autoclave Mod. 2283, Egloff & Co. Niederrohydorf, Switzerland)

6. Shaking incubator

7. Water-bath incubator

8. VORTEX reagent mixer (e. g. VORTEX GENIE, Bender-Hobein, Zürich, Switzerland)

9. Diluter (e. g. Micro Lab 1000, Hamilton, Bonaduz, Switzerland)

10. Photometer equipped with flow cell

11. Programmable calculater or personal computer

Procedure

1. Maintenance of the Strain Culture

Test organism: Streptococcus faecalis (ATCC 8043).

a. Strain cultivation: in slant tubes in Micro-Assay-Culture agar

1) Subculture: monthly; weekly transfer to another slant agar from which the inoculum can be taken.
2) Incubation: 24 h at 37° C.
3) Storage at 4° C.

b. Alternatively using deep frozen ampoules:

1) Strain cultivation: Cell suspension in nutrient broth (see 'Reagents: Micro Inoculum Broth'). Cell density approximately 3-5 x 108/ml; freeze in liquid nitrogen in aliquots of 1.5 ml.
2) Storage: under liquid nitrogen

2. Preparation of the Inoculum

a. Nutrient solution: Micro Inoculum Broth (MIB)

1) Inoculation: one loopful of slant culture in 10 ml MIB
2) Incubation: 16 - 18 h at 37° C
3) Inoculation (from one to the other): Dilution 1 :20 in MIB
4) Incubation: 6 h at 37° C while shaking

b. Alternatively:

1) Inoculation: Thaw one ampoule and dilute in 10 ml MIB
2) Incubation: 6 h at 37° C while shaking

c. Washing of cells: 4 times in physiological saline solution, centrifuge 5 minutes at 3500 rpm

Absorbance of inoculum: 0.4 to 0.7 at 580 nm (1 cm path length)

3. Preparation of the Samples of Premixes and Final Feed

a. Grind the solid test material to a fine powder in a mortar or coffee grinder.

b. Accurately weigh 1-20 g of sample. Mix with 50-100 ml of distilled water and add 3 ml of ammonia 2% per 100 ml. Add enough sodium ascorbate 10% solution of produce a final concentration of about 0.1 % and shake this mixture for 45 minutes at 45° C or place it in an autoclave and heat at 120° C for 15 minutes. After cooling to room temperature adjust to pH 6.8 with sodium hydroxide or hydrochloric acid and make up to a known volume. Filter or centrifuge through a foldung filter (e. g. Schleicher and Schüll no. 597-1/2).

c. Dilute with distilled water and with sodium ascorbate 10% to a final concentration of 0.1 %. The estimated content of folic acid in this sample extract should correspond to about 20ng/ml. If the concentration in the extract is not known, test several dilutions.

4. Microbiological Assay

a. Three different samples concentrations and three standard concentrations are prepared as follows.

b. The test tubes are filled with 10 ml of the nutrient solution (folic acid assay medium, single strength; see 'Reagents') and with 0.025, 0.05 and 0.1 ml of the sample extract or the standard solution by means of a diluter. Six control test tubes do not contain additive. The tubes are then steamed for 10 minutes at 120° C in an autoclave (for alternatively 30 minutes in a pressure cooker at 100° C).

c. After cooling to room temperature all test tubes, with the exception of 2 blank tubes without additive, are each inoculated with 1 drop of inoculum (see 'Preparation of the Inoculum').

d. All tubes are incubated for 16-18 hours at *37*° C in a water-bath incubator.

5. Turbidity Measurement

a. The turbidity of the grown cells is measured at 580 nm.

b. The assay is not valid if there are distinc signs of growth in the inoculate blanks. The test conditions (medium, water etc.) must then be examined.

Calculation:

1. Evaluation of the folic acid content by the 6-point method according to Cavalli-Sforze (see appendix or reference) with the aid of a programmable calculator or a personal computer.

2. The assay is not valid if the dose-response curves of sample and control differ significantly from parallelity. Different extraction methods must be tried in such
cases.

3. Test every sample in two independent assays. Correspondence of repeated values of folic acid should be within $\pm$10% of the mean. Otherwise at least one more repeat assay is necessary.

4. Evaluation according to the 4-point method: When slope of a sample curve does not run parallel in the upper or lower part, then and evaluation of the parallel range can be carried out with the 4-point method (see appendix or reference 'Cavalli-Sforza'). Repetition is necessary in such cases.

Variations or exceptions for different products:

Special advice or precautions:

Control checks of the assay should be carried out for every new lot of nutritient broth or every new batch of frozen cells and at least once a year by recording a dose-response curve. The test concentration of folic acid (20ng/ml) must be in the linear range of the semi-logarithmic curve.

Determination of α- Tocopherol acetate in Vitamin E 50% by Gas Chromatography

Assay Parameter: Vitamin E

Principle: Gas chromotography using dotriacontane as the internal standard.

Internal standard solution. Dissolve 0.20 g of *dotriacontane* in *hexane* and dilute to 100.0 ml with the same solvent.

Test solution. Weigh accurately a quantity of the preparation to be examined corresponding to about 0.100 g of α-tocopherol acetate in a 250 ml conical flask. Add 20 ml of 1 *M hydrochloric acid* and treat with ultrasound at 70 °C for 20 min. Add 50 ml of *ethanol* and 50.0 ml of the internal standard solution and thoroughly mix the two phases for 30 min. Allow to separate, and use the upper layer.

Reference solution. Dissolve 0.100 g of alpha-tocopherol acetate in the internal standard I solution and dilute to 50.0 ml with the internal standard solution.

The chromatographic procedure may be carried out using:

- A silanised glass column 2.0 m to 3.0 m long and 2.2 mm to 4.0 mm in internal diameter packed with *diatomaceous earth for gas chromatography* (125 µm to 150 µm or 150 µm to 180 µm), silanised with dimethyldichlorosilane and impregnated with 1 per cent *m/m* to 5 per cent *m/m* of *poly(dimethyl)siloxane;* a plug of silanised glass wool is placed at each end of the column,

- *Nitrogen for chromatography* as the carrier gas at a flow rate of 25-90 ml/min,
- A flame-ionisation detector,

Maintaining the column at a constant temperature between 245 °C and 280 °C and the injection port and the detector each at a constant temperature between 270 °C and 320 °C. Set the temperature of the column and the flow rate of the carrier gas in such a manner that the required resolution is achieved.

Make the injections directly onto the column or via an injection port (preferably glass-lined) using an automatic injection device or some other reproducible injection method. Measure the peak areas by electronic integration.

Resolution. Inject 1 µl of the reference solution. Repeat this operation until the response factor (RF) determined as described below is constant to within ± 2 per cent. The resolution *(R_S)* between the dotriacontane peak and the α-tocopherol acetate peak is at least 1.4.

Interference test. Weigh accurately a quantity of the substance to be examined corresponding to about 0.100 g of α-tocopherol acetate in a 250 ml conical flask. Add 20 ml of *1 M hydrochloric acid* and treat with ultrasound at 70 °C for 20 min. Add 50 ml of *ethanol* and 50 ml of *hexane* and thoroughly mix the two phases for 30 min. Allow to separate. Inject 1 µl of the upper layer and record the chromatogram, choosing an attenuation such that the height of the peak corresponding to α-tocopherol acetate is greater than 50 per cent of the maximum recorder response; during the recording, change the attenuation so that any peak appearing with the same t_R value as for dotriacontane is recorded with a sensitivity at least eight times greater than for the α-tocopherol acetate peak. If a peak with a height of at least 5 mm for a recorder paper width of 250 mm is detected with the same t_R value as for dotriacontane, use the corrected peak area $S'_{D(corr.)}$ for the final calculation.

$$S'_{D(corr)} = S'_D - \frac{S_I \times S'_T}{f \times S_{TI}}$$

S'_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_I = area of the interfering peak (same t_R value as that of the internal standard) in the chromatogram obtained in the interference test,

S'_T = area of the peak corresponding to α-tocopherol acetate in the chromatogram obtained with the test solution,

S_{TI} = area of the peak corresponding to α-tocopherol acetate in the chromatogram obtained in the interference test,

f = factor by which the attenuation was changed.

Inject 1 µl of the reference solution and record the chromatogram, choosing an attenuation such that the peak corresponding to α-tocopherol acetate is greater than 50 per cent of the maximum recorder response. Measure the areas of the peaks corresponding to α-tocopherol acetate (S_T) and to dotriacontane (S_D) and

determine the response factor (RF) as described below. Inject 1µl of the test solution in the same manner. Measure the areas of the peaks corresponding to α-tocopherol acetate (S'_T) and to dotriacontane (S'_D).

Determine the response factor (RF) for α-tocopherol acetate in the chromatogram obtained with the reference solution from the areas of the peak corresponding to α-tocopherol acetate and the peak corresponding to dotriacontane using the expression:

$$\frac{S_D \times m_T}{S_T \times m_D}$$

Calculate the percentage content of α-tocopherol acetate using the expression:

$$\frac{100 \times S'_T \times m_D \times RF}{S'_{D(corr)} \times m}$$

S_D = area of the peak corresponding to the internal standard in the chromatogram obtained with the reference solution,

$S'_{D(corr.)}$ = corrected area of the peak corresponding to the internal standard in the chromatogram obtained with the test solution,

S_T = area of the peak corresponding to α-tocopherol acetate in the chromatogram obtained with the reference solution,

S'_T = area of the peak corresponding to α-tocopherol acetate in the chromatogram obtained with the test solution,

m_D = mass of the internal standard in the test solution and in the reference solution in milligrams,

m_T = mass of a-tocopherol acetate in the reference solution in milligrams,

m = mass of the substance to be examined in the test solution in milligrams.

Determination of Vitamins B_1, B_2, B_6 and Niacin in Concentrates and Premixes by Liquid Chromatography

Assay Parameter: Vitamin **B_1, B_2, B_6** and Niacin

Principle: HPLC

Products to which applicable: Concentrates and premixes

Assay range: 2-100%

Origin of Method (published or unpublished reference):

1. Cargill Incorporated Central Analytical Services
2. Taille, Thornhill, Toomay, HPLC "Analysis of B-Vitamins in Enrichmen Concen- trates" Pre-Publication Manuscript.
3. Wills, Shaw, Day, "Analysis of water Soluble Vitamins by HPLC", Journal of Chromatographic Science, Vol. 15, 262, 1977.
4. "Official Methods of Analysis of the AOAC", 12th Edition, #43-024 p. 823.

Reagents (and sources where applicable)

1. Methanol (HPLC Grade)
2. Acetic acid (AR Grade)
3. Hexane sulphonic acid (Eastman Kodak)
4. Sodium citrate (Reagent Grade)
5. Pyridoxine HCI (Thiamin, Niacin, Riboflavin) standards (US Biochemical Corporation)

Apparatus (and sources where applicable)

1. HPLC with UV absorbance detector (operating at 280 nm) and 20 microlitre fixed loop injector (See Special Advice #1).
2. Reverse phase column C18 (E. Merck & Assoc.)
3. Volumetric flasks and pipets
4. Balance capable of weighing ±0.0001 gm
5. #42 Whatman filter papers

Standard Solutions

1. 10/90 methanol/water
2. Mobile phase 0.005M Hexane sulfonic acid, 0.025 M Sodium citrate in a 10% solution of methanol in water. Adjust pH of final solution to 3.5 with concentrated acetic acid (See Special Advice #4)

Procedure

1. Weigh an amount of sample calculated to contain 50 mg of pyridoxine HCI (or any other a.m. vitamin) into a 50 ml volumetric flask.

2. Add about 40 ml 10% methanol in water, swirl, and place in ultrasonic bath for 5 minutes. Dilute to mark with 10% methanol in water and mix.

3. Prepare a standard vitamin solution in the same manner as the sample.

4. Make 10% dilutions of standard and samples, with 10% methanol in water.

5. If the sample contains a carrier which is insoluble in 10% methanol, filter a portion of the sample through the #42 Whatman filter paper prior to injection into the LC. Vitamin peaks in the sample and standard should be similar in height 60 to 80% of full scale on an attenuation of 1.0 AUFS or less.

Calculations

$$\% \text{ Vitamin in sample} = \frac{HT\ Samp}{HT\ Std} \times \frac{WT\ Std}{WT\ Samp} \times \frac{DF\ Samp}{DF\ Std} \times 100$$

Where HT Samp = Average peak height of the sample vitamin peak
HT Std = Average peak height of the standard vitamin peak
WT Samp = Weight of the sample in grams
WT Std = Weight of the standards in grams
DF Samp = Dilution factor of the sample. Example - If the sample is dissolved in 100 ml and then diluted tenfold the dilution factor is 100 X 10 = 1000
DF Std = Dilution factor of the standard

Variations or exceptions for different products:

Special advice or precautions:

1. If other vitamins are to be determined simultaneously with pyridoxine the detector should be 280 nm. Thiamine alone should be determined at 254 nm.
2. Ion-pairing - mobile phase should be fresh weekly
3. The LC system should not be shut down with Ion Pairing solution in the column. Purge with 25 ml of 50% methanol/water before stopping overnight.

4. Thiamine or riboflavin will give a very broad peak. A mobile phase with higher methanol concentration should be used to cause quicker elution of these two vitamins. Thiamine, 15-20% methanol, 85-80% water-citrate-ion-pair. Riboflavin 30- 40% methanol, 70-60% water-citrate-ion-pair. A greater concentration of ion-pairing reagent will also give a longer retention time. All should have a final pH of 3.5 before use on the HPLC.

5. Precision of the method is ± 2%. If greater precision is required an internal standard must be used.

6. Methanol is flammable and poisonous if ingested. May be harmful if inhaled or absorbed through skin. Keep away from heat, sparks and open flame. Keep container closed. Use with adequate ventilation. Avoid contact with eyes, skin or clothing.

7. Hydrochloric acid and acetic acid can be fatal if swallowed and can cause severe burns. Vapore harmful. Do not get in eyes, on skin, or on clothing. Use with adequate ventilation. In case of contact, flush eyes with plenty of water for at least 15 minutes. Contact a physician immediately.

Microbiological Assay of Vitamin B_{12} in Complete Feeds and Premixes

Assay Parameter: Vitamin **B_{12}**

Principle: Microbiological, turbidimetric

Product(s) to which applicable: Complete Feeds and Premixes

Assay range: Above 20 ng/kg

Coefficient of variation: 10%

Origin of Method (published or unpublished reference):

Cavalli-Sforza, L.: Biometry, 5th ed. G. Fischer Publishing Co., Stuttgart (1980)

Skeggs, H.R.: In P. Gyorgy, W. N. Pearson, editors, The Vitamins; 2nd ed., volume VII, pp 282-284: Academic Press, New York and London (1967)

Analytical Methods for Vitamins and Carotenoids in Feeds, F. Hoffmann -La Roche & Co. AG, CH-4002, Basel, Switzerland

Reagents (and sources where applicable):

As far as possible, all chemicals are of analytical grade.

1. Hydrochloric acid 0.1 mol/l
2. Sodium hydroxide 0.1 mol/l
3. 96% ethanol, non-methylated
4. Potassium cyanide
5. Micro-Assay-Culture agar (Difco Laboratories. Detroit USA; no 0319-02): prepare according to the instructions of the manufacturer.
6. Micro Inoculum Broth (MIB; Difco, no 0320-02): prepare according to the instructions of the manufacturer
7. B_{12} Assay Medium USP (test nutrient solution from Difco, no. 0457-15): prepare according to the instructions of the manufacturer (single strength)
8. Physiological saline solution: 8.5 g sodium chloride dissolved in water made up to 1 litre or Ringer's solution (Merck, Darmstadt, FRG; no. 15525: prepared according to the instructions of the manufacturer).

Sterilize for 30 minutes at 120°C and 1 bar

Standard Solutions

Cyanocobalamin stock solution (2 µg/ml): Dissolve 23 mg cyanocobalamin (USP reference standard, content ca. 86.6%) in 50 ml dist. water and make up to 100 ml with ethanol. Equilibrate at 20°C and make up to 100 ml with ethanol. The shelf life of this solution is 4 weeks when stored in a dark place at 4°C.

To prepare the final standard solution (2 ng/ml) dilute the stock solution 1 to 1000 by volume with distilled water.

Apparatus (and sources where applicable)

1. Food processor (e.g. Moulinex, France) or grinder (e.g. coffee grinder MX *J* 32/MXK; Braun AG, Frankfurt/M , FRG)
2. Autoclave (e.g. Fedegari S.P .A., Albuzzano [PV], Italy)
3. Centrifuge (e.g. laboratory centrifuge 4000 rpm, Heraeus-Christ GmbH, Osterode, FRG; with 40-ml centrifuge tubes with ground stoppers
4. pH-meter with glass electrode
5. Steamer or small autoclave (e.g. EGRO autoclave 2283, Egloff & Co. Niederrohrdorf, Switzerland)
6. Shaking incubator
7. Water-bath incubator
8. VORTEX reagent mixer (e.g. VORTEX-GENIE, Bender-Hobein, ZOrich, Switzerland)
9. Diluter (e.g. Micro Lab 1000, Hamilton, Bonaduz, Switzerland)
10. Photometer equipped with flow cell
11. Programmable calculator or personal computer

Procedure

1. Maintenance of the Strain Culture

Test organism: Lactobacillus leichmannii (ATCC 7830)

a. Strain cultivation: Stab culture in Micro-Assay-Culture agar

1) Subculture: monthly; transfer weekly to another stab culture from which the inoculum can be taken
2) Incubation: 24 h at 37°C
3) Storage: at 4°C

b. Alternatively using deep frozen ampules:

1) Strain cultivation: cell suspension in nutrient broth (See "Reagents: Micro- Assay-Culture agar"). Cell density approx. 5 x 10^8/ml; freeze in liquid nitrogen in aliquots of 1.0 ml
2) Storage: under liquid nitrogen

2. Preparation of the Inoculum

a. Nutrient solution: Micro Inoculum Broth (MIB)

1) Inoculation: one loopful of stab culture in 10 ml
2) Incubation: 16-18 h at 37°C
3) Transfer Dilution 1:10 in MIB
4) Incubation: 6 h at 37°C while shaking

b. Alternatively:

1) Inoculation: thaw one ampoule and dilute in 10 ml MIB
2) Incubation: 6 h at 37°C while shaking

c. Washing of cells: 3 times in physiological saline solution, centrifuge 5 minutes at 3500 rpm

1) Absorbance of inoculum: 0.4 to 0.7 at 580 nm (1 cm path length)

3 . Preparation of the Samples of Premixes and Feedstuffs

Grind the solid test material to a fine powder in a mortar or coffee grinder.

Accurately weight about 10 g of sample. Mix with 50 ml water and add one spatula tip of potassium cyanide, as stabilizer for the extraction of the bound vitamin B_{12}. Shake the batch for 45 minutes at 45°C or alternatively autoclave at 120°C for 10 minutes. After cooling to room temperature adjust exactly to a known volume and filter through a folded filter (e.g. Scr11eicher and Schüll no. 5971/2) or centrifuge. Adjust an aliquot of at least 5 ml of the filtered extract to a pH of 6.0 with sodium hydroxide or hydrochloric acid.

Dilute with dist. water so that the estimated content corresponds to that of the standard (2 ng/ml). If the concentration of the extract is not known, test several dilutions.

4 . Microbiological Assay

Three different sample concentrations and three standard concentrations are prepared as follows.

The test tubes are filled with 10 ml of the nutrient solution (B_{12} assay medium, single strength; see "Reagents: Micro Inoculum Broth") and with 0.025,0.05 and 0.10 ml of the sample extract or the standard solution by means of a diluter. Six blank test tubes do not contain additive. The tubes are steamed for 30 minutes at 100°C or autoclaved for 5 minutes at 121°C.

After cooling to room temperature, all test tubes with the exception of 2 blank tubes, are each inoculated with 1 drop of inoculum (see "Preparation of the Inoculum").

All tubes are incubated for 16-18 hours at 37°C in a water-bath incubator.

5. Turbidity Measurement

The turbidity of the grown cell suspension is measured at 580 nm.

The assay is not valid if there are distinct signs of growth in the inoculated blanks. The test conditions (medium, water etc.) must then be examined.

Calculation:

Evaluation of the vitamin B_{12} content by the six-point method according to Cavalli- Sforza (see appendix or references) with the aid of a programmable calculator or a personal computer.

The assay is not valid if the dose-response curves of sample and control differ significantly from parallelity. Different extraction methods must be tried in such cases.

Test every sample in two independent assays. Correspondence of repeated values of vitamin B_{12} should be within ± 10% of the mean. Otherwise the assay must be repeated at least once more.

Evaluation according to the four-point method: When the slope of a sample curve does not run parallel in the upper or lower part, then method (see appendix or reference "Cavalli-Sforza"). In any case repetition is necessary.

Variations or exceptions for different products:

Special advice or precautions:
Control checks of the assay should be carried out for every new lot of nutrient broth or every new batch of frozen cells and at least once a year by recording a dose-response curve. The test concentration of vitamin B_{12} (20 ng/ml) must be in the linear range of the semi-logarithmic curve.

Determination of Vitamin D_3 in Complete Feeds and Premixes with HPlC

Assay Parameter: Vitamin **D_3**

Principle: HPLC w/UV Detection

Product(s) to which applicable: Complete Feeds and Premixes

Detection Limit: 1000 IU D_3/kg

Assay range: Above 1000 IU vitamin D_3/kg.

Coefficient of variation:

Vitamin D_3 Content (IU/kg)	Range (estimated)
1 000- 5 000	±1000 IU/kg
5 000- 20 000	± 20%
20 000- 100 000	±15%
above 100 000	±10%

Origin of Method (published or unpublished reference): U. Manz and K. Philipp

Reagents (and sources where applicable)

1. Butylated hydroxytoluene (BHT)
2. Dioxane
3. 2-Propanol (isopropyl alcohol)
4. Ethanol, absolute
5. Diethyl ether, peroxiae-free
6. n-hexane
7. Iso-octane
8. Methanol
9. Potassium hydroxide 50% aqueous (100 9 potassium hydroxide pellet dissolved in distilled water and made up to 100 ml)
10. Sodium chloride 10% aqueous solution

Standard Solution:

(400 IU Vitamins D_3/ml): Dissolve 20 mg cholecalciferol (vitamin D_3) cryst. For calibration in 20 ml n-hexane and dilute 1: 1 00 with n-hexane.

Apparatus (and sources where applicable)

1. Round-bottomed flask with reflux condenser
2. Water-bath or electrothermal mantle
3. Rotary evaporator
4. 2 HPLC-lines consisting of the modules:

a. Injection valve or auto sampler
b. Mono or double piston pulse-free pump
c. Variable or fixed wavelength UV-detector
d. Integrator
e. Recorder

Procedure

1. Grind the solid test material to a fine powder and mix well.
2. Accurately weigh the following amounts of test material into a 250-ml round-bottomed flask.

Content IU D_3/kg	Sample Size Gram
1 000-10 000	20
10 000-50 000	10
> 50 000	5

3. Add 70 ml ethanol and 20 ml potassium hydroxide solution (50%) and bring the content of the flask to boiling point (boiling water-bath, electrothermal mantle). Saponify for 20 minutes under reflux. Agitate from time to time. At the end of saponification, cool to about 40° C.
4. Transfer the contents of the flask quantitatively into a seperatory funnel (500 ml) and subsequently wash the condenser and the round-bottom flask with 100 ml dist. water, adding the washings to the separatory funnel. Extract the saponification solution with 120 ml ethyl ether by vigorously shaking the two solutions for about half a minute. Let the mixture stand until the two phases have completely separated. Transfer the lower layer (saponification solution) into a second separatory funnel containing 120 ml ethyl ether and repeat the extraction step.
5. Combine the two ether phases and wash them until neutral with:
    a. 100 m110% sodium chloride solution
    b. 100 ml dist. water
    c. 100 ml dist. water containing 10% ethanol
    d. 100 ml dist. water
6. Add 100 mg BHT to the ether extract and evaporate the solvent in a round-bottom flask with the aid of a rotary evaporator under partial vacuum and at a water-bath temperature of about 40° C. Evaporate the residual water by repeated additions of abs. ethanol.

7. Dissolve the residue in 5 ml methanol (volumetric flask). Inject 0.5 ml of the methanol solution into semipreparative HPLC column (see 'For purification of the extract') and collect the fraction containing the vitamin D_3. Under the conditions mentioned it appears between the 24th and 32nd minute (check the elution time with a standard vitamin D_3 solution in methanol). Evaporate this fraction (about 16 ml) to dryness in a small conical flask (50 ml) and dry the residue by addition of successive portions of ethanol.

8. With samples with a declared content of above 100 000 IU vitamin D_3/kg the step with the semipreparative HPLC column may be omitted.

9. Add 1.0 ml iso-octane, stopper the flask and dissolve the residue in the solvent. Inject 0.1 ml (100 µl) into an analytical HPLC column (for conditions see 'HPLC conditions for quantitative determination of D_3) and determine the vitamin D peak by area or by hight, comparing it to a peak of a standard solution of vitamin D_3 (containing about 400 IU vit. D_3/ml n-hexane).

Conditions for High Performance Liquid Chromatography:

1. For purification of the extract:
   a. Column and stationary phase: HIBAR, LiChrosorb RP-8, 7µm, column length: 250 mm, inner diameter 10 mm.
   b. Mobile phase: Methanol/water (92:8) isocratic.
   c. Flow: 2.0 ml/min.
   d. Temperature: ambient.
   e. Detection: UV-detection at 264 nm.
   f. Run time: minimal 60 min. before next injection.
2. For quantitative determination of vitamin D_3:
   a. Column and stationary phase: HIBAR, LiChrosorb Si 60,5 µm, column length: 250 mm, inner diameter 4 mm.
   b. Mobile phase: n-hexane containing 5 percent dioxine and 0.5 percent 2- propanol, isocratic.
   c. Flow: 1.5 ml/min.
   d. Temperature: ambient.
   e. Detection: UV-detection at 264 nm.
   f. Retention time: 11-11.5 min.
   g. Run time: minimal 30 min.

Calculations:

1. The area counts or the peak heights of the vitamin D_3 peaks in the sample solution are compared with the corresponding areas or heights of the standard solution.

2. Conversion: 1 International unit (IU) vitamin D_3 corresponds to 0.025 µg cholecalciferol:

Microbiological Assay for Biotin in Complete Feeds and Premixes

Assay Parameter: **Biotin**

Principle: Microbiological Turbidimetric

Product(s) to which applicable: Complete Feeds and Premixes

Assay range: Above 1 0 µg/kg

Coefficient of variation: ± 10%

Origin of Method (published or unpublished reference):

Barton-Wright, E. C.: Practical Methods for the Microbiological Assay of the Vitamin B Complex and the Essential Amino Acids, Ashe Laboratories Ltd., London (1952)

Media for Microbiological Assay of Vitamins and Amino Acids, Difco Laboratories, Detroit (1964)

Baker, H., Frank, O.: Clinical Vitaminology, Methods and Interpretation, John Whiley and Sons, New York, London (1968)

International Journal of Vitamin and Nutrition Research, 46 (1976), 314-321

Frigg, M., Brubacher, G.: Biotin Deficiency in Chicks Fed a Wheat-Based Diet

Analytical Methods for Vitamins and Carotenoids in Feeds, F. Hoffmann-La Roche & Co. AG, CH-4002, Basel, Switzerland

Reagents (and sources where applicable)

As far as possible, all chemicals are of analytical grade.

1. Citrate buffer pH 5.5: Dissolve 21 g citric acid in dist. Water and adjust the volume to 1 litre (solution 1). Dissolve 37.5 g disodium phosphate (e. g. Merck, Darmstadt, FRG) in dist. Water and adjust the volume to 1 litre (solution 2). Mix 44 ml of solution 1 and 56 ml of solution 2; dilute to 1 litre with water.
2. Papain solution: Dissolve 500 mg papain (EC 3.4.22.2; 30,000 USP-U/mg; e. g. Merck, no. 7144) in 50 ml citrate buffer. Prepare freshly before use.
3. Volatile preservative: Mix 1 volume 1,2-dichloromethane, 1 volume chlorobenzene and 2 volumes 1-chlorobutane (e. g. Merck)
4. Sulphuric acid, 1 mol/l (e. g. Titrisol, Merck)
5. Sodium hydroxide 20%

(Vitamin K3)

Determination of Combined Forms of Menadione in Complete Feeds, Concentrates and Premixes by modified 2,4-Dinitrophenylhydrazine Method

Assay Parameter: Menadione

Principle: Spectrophotometric

Product(s) to which applicable: Feedstuffs, concentrates and premixes

Detection Limit: 1 ppm

Coefficient of variation: 10-20 %

Origin of Method (published or unpublished reference):

Gaudiano, A., Bellomonte, G., et al., Revista Della Scoeita Italiana di Scienza dell'Alimentazione 6 (3) 193 (1977)

Reagents (and sources where applicable)

1. 96% *(v/v) Ethanol.* Dilute absolute ethanol with H_2O.
2. *40% Ethanol* Dilute Reagent 1 with H_2O to bring to 40%.
3. *10% Tannin Solution* Prepare by weighing out 10 g tannin and dissolving in H_2O. Bring final volume to 100 mL.
4. *10% Sodium Carbbonate Solution* Prepare--by weighing out 10 g Na_2CO_3 and dissolving in H_2O. Bring final volume to 100 mL.
5. *2-4 Dinithrophenylhydrazine (DNPH)* Dissolve 40 mg DNPH in about 40 mL boiling absolute ethanol, allow to cool and transfer to a 50-mL volumetric flask. Add 1 mL concentrated HCI (d = 1.19) and make up to volume with absolute ethanol. Prepare immediately before use.
6. *Ammoniacal Ethanol* Mix one volume of absolute ethanol with one volume of concentrated NH_4OH (d = 0.91).
7. *Menadione Standard.* Dissolve 20 mg menadione in 1,2-dichloroethane and dilute to 200 mL with the same solvent. Dilute aliquots of this stock solution with 1,2- dichloroethane to obtain a series of solutions with menadione concentrations between 2 and 10 µg/mL. These solutions must be prepared immediately before use.

Apparatus (and sources where applicable)

1. Mechanical shaker
2. Centrifuge

3. Separatory funnels 4. Rotary evaporator
4. Spectrophotometer
5. Laboratory glassware

Procedure

All operations must be carried out away from direct light, using apparatus of amber glass where necessary.

1. *Test Sample.* From the finely divided sample, take a test sample according to the presumed menadione content, for example, 0.1 - 5.0 g for concentrates and premixes, 20 - 30 g for feedstuffs. Transfer the sample to a 250-mL flask with ground stopper without delay.

2. *Extraction.* Add to the test sample exactly 96 mL 40% ethanol (Reagent 2) and shake mechanically for 15 min. at room temperature. Then add 4.0 mL tannin solution (Reagent 3), mix, transfer the extract into a centrifuge tube, centrifuge at 3000-5000 rpm and decant.

   Place 20-40 mL, accurately measured, of the extract in a 250-mL separatory funnel add 50 mL 1,2-dichloroethane, mix and add by pipette 20 mL Na_2CO_3 solution (Reagent 4). Shake vigorously for 30 sec., collect the dichloroethane phase in a 100-mL separatory funnel. Add 20 mL H_2O, shake again for 15 sec. Collect the dichloroethane phase and remove traces of water with strips or filter paper.

   For concentrates and premixes, take an aliquot of the extract and dilute with 1,2- dichloroethane to obtain a menadione concentration of 2-10 µg/ml. For feedstuffs, evaporate an aliquot of the extract to dryness under reduced pressure in a rotary vacuum evaporator under nitrogen on a water bath at 40° C. Rapidly treat residue with 1 ,2.dichloroethane to obtain a solution containing 2-10 µg/mL.

3. *Hydrazone Formation.* Transfer 2.0 mL of the extract obtained in Step 2 to a 10-mL volumetric flask and add 3.0 mL DNPH (Reagent 5), securely stopper the flask with a cork or Teflon stopper to prevent evaporation, and heat for 2 hr. at 70° C on a water bath. Allow to cool, add 3.0 mL ammoniacal ethanol (Reagent 6), mix, make up to volume with absolute ethanol and mix again.

4. *Measurement* of *the Optical Density.* Measure the optical density of the blue-green colored complex with spectrophotometer at 635 nm, using a reagent blank obtained by treating 2.0 mL 1,2-dichloroethane as indicated in Section C3. Determine the quantity of menadione by reference to a calibration curve established for each sereis of analyses.

5. *Calibration Curve.* Treat 2.0 mL of menadione standard solutions (Reagent 7) as described in Section C3. Measure the optical density as indicated in Section C4. Plot the calibration curve with the optical density value as ordinates and the corresponding quantities of menadione (in Ilg) as abscissae.

6. *Calculation* of *Results.* Calculate the menadione content of the sample by taking account of the weight of the test sample and of the dilutions carried out in the cours of the analysis.

   $$\frac{\text{µg Menadione from standard curve X 100}}{\text{µg of Sample represented in final dilution}} = \% \text{ Menadione}$$

   Menadione values obtained can be converted to other forms using the same conversion factors given in Method I.

7. Reproducibility. The difference between the results of two parallel determinations carried out on the same sample must not exceed:

   a. 20% in relative value, for menadione contents less than 10 ppm

   b. 2 ppm, in absolute value, for content between 10 and 14 ppm

   c. 15% in relative value, for contents between 14 and 100 ppm

   d. 15 ppm, in absolute value, for contents between 100 and 150 ppm

   e. 10% in relative value, for contents greater than 150 ppm

Variations or exceptions for different products:

Special advice or precautions: Avoid exposing menadione or its solutions to light during the assay.

Determination of Menadione by Titration (Vitamin K3)

Weigh accurately about 150 mg of Menadione ($C_{11}H_8O_2$) or equivalent amount of Menadione derivate, and transfer to a 150-ml flask. Add 15 ml of glacial acetic acid and 15 ml of 3 N hydrochloric acid, and rotate the flask until the Menadione is dissolved. Then add about 3 g of zinc dust, close the flask with a stopper bearing a Bunsen valve, shake, and allow to stand in the dark for 1 hour, with frequent shaking. Rapidly decant the solution through a pledget of cotton into another flask, immediately wash the reduction flask with three 10-ml portions of freshly boiled and cooled Water, add 0.1 ml of orthophenanthroline TS, and immediately titrate the combined filtrate and washings with 0.1 N ceric sulfate VS. Perform a blank determination, and make any necessary correction. Each ml of 0.1 N ceric sulfate is equivalent to 8.609 mg of $C_{11}H_8O_2$.

Assay of Calcium D-Pantothenate (Calpan) in pure substance by Potentiometric Titration

Assay Parameter: Calcium D-Pantothenate (Calpan)

Principle: Potentiometric Titration

Product(s) to which applicable: Calcium D-Pantothenate

Reagents

1. Acetic acid p.a. (100%)
2. Perchloric acid 0.1 mol/l

Procedure

Dissolve 180 - 200 mg (accurately weighed) in 75 ml acetic acid (100%). Titrate potentiometrically with perchloric acid 0.1 mol/l using a combined glass electrode.

Calculation

Each ml of perchloric acid 0.1 mol/l is equivalent to 23.827 mg