Method of analysis for Vitamins

Fat-soluble vitamins, such as vitamins E, D, and A, and water-soluble vitamins, such as vitamins C, B6, B2, B1, and B1, have been analyzed.

Method:

Vitamin B1 (Thiamine)

The following procedure used for determination of thiamine in premixes and compound feeds was elaborated on the basis of the article published by Rubaj et al.

- Principle:
  Vitamin B1 is extracted with hydrochloric acid of 0.1 mol/l and next oxidized to thiochrome and marked with the use of high performance liquid chromatography (HPLC) with a fluorescence detection or PDA detector.
- Reagents and Solvents: All reagents and solvents should be of analytical grade: chloroform,methanol; hydrochloric acid, c=0.1 mol/l; Trichloroacetic acid, 50%; Sodium hydroxide, 15%, Water, Saturated isobutanol, Potassium hexacyanoferrate (III), Vitamin B1 standard, Takadiastase, Sodium acetate.
- Apparatus:
  Laboratory shaker, Centrifuge, Water bath with condenser, HPLC set with fluorescence detector/ PDA detector.
- Procedure:
  Thiamine was extracted from the examined feed sample with 0.1 M hydrochloric acid at 100°C for 30 minutes. In case of compound feedingstuffs 10% taka-diastase solution was added to the samples, and then samples were incubated at 37°C for 17 hours. Afterwards thiamine was oxidized to thiochrom by 1% alkaline K3Fe(CN)6.
- Chromatography:
  Column: 25 cm x 4.6 mm
  Mobile phase: Chloroform and methanol, 90+10 (v/v)
  Column temperature: 25 ºC
  Flow rate: 2.0 ml/min
  Injection: 20 µl
  Detector: Fluorescence/PDA, Ex λ= 365, Em λ=435
- Calculation : External standard, peak area, linear regression
  Special Comment
  This method was applied for the quantification of total content of thiamine in compound Feeding stuffs as well as added thiamine in the form of hydrochloric or nitrate salt.

Vitamin B2 (Riboflavin)

The following procedure for determination of riboflavin in premixes and compound feeds was elaborated on the basis of the article published by Rubaj et al.

- Principle:
  Riboflavin was extracted from a feed sample in autoclave with 0.1M sulphuric acid. The ester bonds with phosphoric acid were hydrolyzed by the Taka-diastase enzyme. Riboflavin Using High Performance Liquid Chromatography (HPLC) for Analyzing Feed Additives content was determined by high performance liquid chromatography (HPLC) with reversed-phase and usage of fluorescence detection.
- Reagents and Solvents: All reagents and solvents should be of analytical grade.Methanol for HPLC, Sulphuric acid, 0.1 mol/l; Sulphuric acid, 30%; Sodium hydroxide, 0.5 mol/l; sodium acetate, 2mol/l, Acetic acid, 99.5%, Citric acid, Taka-diastase, 10% suspension, Vitamin B2 standard.
- Apparatus: Autoclave, Ultrasound bath, HPLC set with fluorescence detector.
- Procedure:
  Riboflavin was extracted from the examined feed sample with 0.1M sulphuric acid, and that solution was boiled for 15 min. at temperature from 110°C to 120°C. After cooling to the room temperature, the whole volume of hydrolysed sample was transferred to a 100 ml measuring flask. Next taka-diastase suspension was added to the flask, which was then placed into a water bath at 45°C for 20 min. The enzymatic reaction was stopped by adding sulphuric acid. The sample solution was next chilled to room temperature, and the volume was corrected to 100 ml by adding 0.1 mol/l sulphuric acid. Afterwards, samples were mixed and filtrated. Extract clean-up was done by adding methanol to the sample and filtration through syringe filter before injection on the column.
- Chromatography
  Column: 25 cm x 4.6 mm
  Mobile phase: Methanol and citric acid 0.2 mg/l (30:70 v/v). That solution was mixed with methanol with ratio 1:1
  Column temperature: 25 ºC
  Flow rate: 0.8 ml/min
  Injection: 20 µl
  Detector: Fluorescence, Ex λ= 453, Em λ=521
- Calculation: External standard, peak area, linear regression
  Special Comment
  Vitamin B2 is sensitive to light, hence all the activities were conducted without any

access of day light (by using amber glass flask or flask covered by aluminum foil).

Canthaxanthin

The following procedure for determination of canthaxanthin in premixes and compound feeds was elaborated on the basis of the article published by Rubaj et al.

- Principle:
  The principle of this method is based on the hydrolysis of a powdered formulation of canthaxanthine with trypsin and pepsin in water solution of ammonia and its purification on the aluminium oxide filled column. The canthaxantine content is determined by high performance liquid chromatography (HPLC) in normal phase with usage of DAD detector – The Most Versatile Method of Chemical Analysis
- Reagents and Solvents: Trypsin 200 FIP – U/g, Pepsin 700 FIP – U/g; Ammonia; N – hexane, Diethyl ether; 99.8% Ethyl alcohol, Acetone, Aluminum oxide, Neutral, Canthaxanthin standard, Chloroform. All reagents and solvents should be of analytical grade.
- Apparatus: ultrasonic bath, Vacuum rotary evaporator, HPLC set with Array's diode detector.
- Procedure:
  The sample is hydrolyzed with an aqueous solution of ammonia at the presence of trypsin and pepsin following extraction with ethyl alcohol and diethyl ether. Purification occurs on the aluminum oxide filled column. The extract prepared in this way should be evaporated, dissolved in the mobile phase, filtered through syringe filters and dosed on the column.
- Chromatography
  Column 4.6 x 250 mm
  Mobile phase n-hexane: acetone 86:14 (v/v)
  Column temperature 25 ºC
  Flow rate 1.3 ml/min
  Injection 20 µl
  Detector DAD λ=446 nm
  Calculation External standard, peak area, linear regression

Methionine hydroxy analog (MHA)

The procedure of analyzing methionine hydroxy analog was developed on the basis of the work by Matyka et al. and the official VDLUFA method.

- Principle:
  Methionine hydroxy analog is extracted from the sample by 10% acetonitrile, and next

hydrolyzed with potassium hydroxide and determined by high performance liquid chromatography (HPLC) with reversed phase and UV detection.

- Reagents and Solvents: Acetonitrile, Orthophosphoric acid, Solution for extraction, Acetonitrile - water 10+90 (V/V), Solution for hydrolysis, 50% potassium hydroxide (w/v); phosphoric acid, 0.01mol/l.
- Apparatus: Centrifuge, HPLC set, Diode array detector.
- Procedure:

  Extract Methionine hydroxy analog from the feed, with the use of 10% Acetonitrile. After centrifuging, perform hydrolysis with potassium hydroxide and next with a solution of orthophosphoric acid. Filter the supernatant through syringe filters and inject on the column. Using High Performance Liquid Chromatography (HPLC) for Analyzing Feed Additives
- Chromatography:

  Column: 25 cm x 4.6 mm

  Mobile phase: Eluent 1 : acetonitrile - phosphoric acid 10+90 (v/v)

  Eluent 2 : acetonitrile - phosphoric acid 23+77 (v/v)

  Column temperature: 25 °C

  Flow rate: 0.8 ml/min

  Injection: 20 µl

  Detector: UV, λ=214 nm

  Calculation: External standard, peak area, linear regression