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Chapter 6

# Using High Performance Liquid Chromatography (HPLC) for Analyzing Feed Additives

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Additional information is available at the end of the chapter

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## 1. Introduction

Feed additives have been widely used in animal nutrition. Recommendations concerning using feed additives, their categories and the description of requirements related to such additives can be found in the regulation (EU) No 1831/2003 [1]. Detailed regulations oblige the entities launching feed additives on the market to specify the methods used to analyse active substances of additives for the needs of official feed control. Official feed control is implemented in order to monitor adequate and safe use of feed additives in animal nutrition. Moreover, controlling feed production in this respect results in improving the quality and safety of animal products for consumers.

HPLC methods have been widely used in the analyses of feed additives, such as vitamins, feed colorants, antioxidants, amino acids and coccidiostats in preparations, premixes and feed mixtures. It is relatively simple to analyse preparations as they are usually composed of a particular active substance and a carrier. A premix is more complex feed consisting of a combination of a dozen different feed additives on a mineral (calcium carbonate) or organic (wheat bran) carrier. Complete feed mixtures used in animal nutrition, produced by combining premixes with feed materials are often greased and subjected to further hydro- or barothermal processing, e.g. pelleting, extrusion or expanding. In order to counteract decomposition of the active substance, feed additives are secured by protective coating, e.g. vitamin A, canthaxanthin, which enhances their durability in the feed matrix. A specific protection of feed additives by protective coating, thermal processing, greasing the feed, varied composition of feed materials in mixtures may hinder the transfer of the active substance into a solution during extraction and purification of the extract.

The key issue becomes selection of a chromatographic column (in a normal phase or reversed phase), mobile phase, detector, as well as optimization the conditions of

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chromatographic separation. In examining feed additives with the use of HPLC methods the most frequently used types are spectrophotometric detection (UV-VIS), detection with the help of diode array and fluorescent detection. The choice of optimal parameters for chromatographic separation is done during validation of the method. The analyses in this respect should be accompanied by an assessment of the method's robustness [2]. A practical way to verify the precision of a method in a laboratory (repeatability) and in interlaboratory studies (reproducibility) is taking advantage of Horwitz equation [3].

Using HPLC methods for examining feed additives was the subject matter of numerous studies on the basis of which official methods of analysing certain feed additives were developed. The studies presented the basic validation parameters for the methods of examining the content of fat-soluble vitamins [4-7], water-soluble vitamins [8,9], coccidiostats [4,10-12], and other feed additives, amino acids, methionine hydroxy analog and antioxidants [13,4,8]. However, in case of carotenoids such as canthaxanthin or apocarotenoic acid ester official methods of examining these additives are still based on spectrophotometric measurement rather than on HPLC methods [13,8].

New requirements have been introduced regarding the validation parameters for the methods of analysing feed additives, e.g. those listed in the regulation No 882/2004 [14], taking into consideration, among others, the uncertainty of measurement. It is necessary to determine the uncertainty of measurement with a particular method in order to interpret adequately the result of examining feed additives in feedingstuffs and to assess acceptable tolerance in compliance with the requirements of the regulation No 939/2010 [15]. The new requirements in this area should be taken into account while validating the methods of testing feed additives in order to solve the problem of interpreting the results.

The aim of the present work was to offer a review of HPLC methods used for analysing active substances in certain feed additives, with regard to current requirements defined in the regulations. In some justified cases the results of the authors' own studies were presented, as well as the procedures for determining vitamins $B_1$ and $B_2$, canthaxanthin and methionine hydroxy analog (MHA). Some validation parameters were presented, such as the limit of the method's quantification (LOQ), linearity of the calibration curve, repeatability, within-laboratory reproducibility (intermediate precision), recovery and the uncertainty of measurement. Also, the results of verifying the developed methods and laboratories participating in proficiency testing (PT) were demonstrated. The ways of quality assurance of the tests in reference to HPLC methods were discussed. The work presents the method of assessing combined standard uncertainty of measurement with the use of experimental approaches based on within-laboratory reproducibility and calculations for the bias of the method on the basis of CRM studies or PT results.

## 2. A review of HPLC methods used to determine feed additives

Feed additives are commonly used in animal nutrition, e.g. in order to supplement the animals' requirement for nutrients (amino acids), useful micro components (vitamins), to

prevent invasive diseases (e.g. coccidiosis – coccidiostats), to reduce oxidation processes of feed's components (antioxidants), to enhance the dietary value and quality in food products of animal origin (amino acids, carotenoids– egg yolk coloration).

In case of some vitamins and antioxidants their maximum content in feed mixtures was determined, e.g. for vitamins A and $D_3$, antioxidants (ethoxyquin, BHA, BHT), carotenoids (canthaxanthin, apocarotenoic acid ester and others). Maximum contents are subject to official control in reference to their conformity with the requirements related to the safety of feedingstuffs. Additionally, a feed manufacturer is obliged to declare the content of feed additives on the label of a premix or feed mixture. Thus, it is necessary to have access to analytical methods for testing the content of feed additives in a wide range of concentrations in the preparations containing additives, premixes and feed mixtures.

Table 1 presents examples of well-known HPLC methods for examining fat-soluble vitamins in feedingstuffs, including the official methods accepted by the European Commission. A commonly used method of preparing a sample for analysing the content of vitamin A is alkaline hydrolysis during which gelatin/sugar cross-linked beadlets which protect vitamin A in the form of retinol acetate are solved and then purified by liquid-liquid extraction. An interesting option for purifying vitamin A extracts from feed mixture with the use of the SPE technique was presented by Fedder & Ploger [7]. The step of alkaline hydrolysis is also used while determining vitamins E and $D_3$. Chromatographic separation of vitamins, except for vitamin $D_3$ where preliminary separation and fraction collection are necessary [5], does not present any serious problems. However, a problem may be posed by the quality and durability of a standard, as well as poor precision resulting from a too low of analytical weight [16]. It is necessary to verify vitamin A standards with the use of a spectrophotometric method [4].

The official methods of determining the content of water-soluble vitamins, such as $B_1$, $B_2$ and $B_6$ are based on spectrophotometric or fluorometric methods [8,13]. The results of analyses using these methods may be biased with errors due to some interferences from other substances in the variable feed matrix. Recently HPLC methods to determine vitamins $B_1$, $B_2$, $B_6$, nicotinic acid and nicotinamide in mineral preparations and mixtures [8], as well as vitamin $B_1$ in feed mixtures and premixes [9] were published (Table 2). Due to the high limit of quantification for vitamin $B_1$ amounting to 5 mg/kg according to Italian Official Method [9], it cannot be used for analysing vitamin $B_1$ in typical feed mixtures to which it is normally added at the amount of 2-4 mg/kg. Moreover, the method quoted above makes it possible to examine vitamin $B_1$ added to feedingstuffs but not the total content of this vitamin, regarding its presence in feed materials. It is thus necessary to have access to chromatographic methods enabling the examination of water-soluble vitamins present in feed materials and added in the form of feed additives. The procedures of HPLC methods of vitamins $B_1$ and $B_2$ developed during the authors' own studies are presented later in the chapter [17,18].

| Analyte, matrix reference | Extraction, extract clean up and chromatography parameters | Performance parameters |
|---|---|---|
| Vitamin A in feedingstuffs and premixes, Commission Regulation, 2009 [4] | Sample hydrolyze with ethanolic KOH, extraction into light petroleum, evaporation and dissolution in methanol, reversed phase HPLC, $C_{18}$ column (250 x 4 mm) 5 µm or 10 µm packing , mobile phase: methanol and water 98+2 (v/v), fluorescence (or UV) detector: excitation 325 nm; emission 475 nm or UV detector (325 nm) | LOQ=2000 IU/kg; $SD_r$ (%): 3.0-8.1; $SD_R$ (%): 6.2-20.0; |
| Vitamin E in feedingstuffs and premixes, Commission Regulation, 2009 [4] | Sample hydrolyze with ethanolic KOH solution, extraction into light petroleum, evaporation and dissolution in methanol, reversed phase HPLC, $C_{18}$ column (250 x 4 mm) 5 µm or 10 µm packing , mobile phase: methanol and water 98+2 (v/v), fluorescence detector: excitation 295 nm; emission 330 nm or UV detector (292 nm) | LOD=2 mg/kg; LOQ=10 mg/kg; $SD_r$ (%): 2.2-4.1; $SD_R$ (%): 4.8-12.7 |
| Vitamin $D_3$ in feedingstuffs and premixes [5] | Feed saponification and extraction with diethyl ether; evaporation and solvation in methanol; reverse phase preparative chromatography; eluat collection with vitamin $D_3$ , evaporation and next solvation in n-hexan or isooctane; normal phase chromatography, column 250 mm x 4 mm, Si-60, 5 µm packing; UV detection at 264 nm; mobile phase: preparative column: methanol -water (92+8)), analytical column – n-hexan – dioxan - isopropanol (94.5+5+0.5) | LOQ=1000 IU/kg; $RSD_r$ to 5000 IU/kg: 1000 IU/kg; 5000-20000 IU/kg:20% 20000-100000 IU/kg: 15%; >100000 IU/kg: 10% |
| Vitamin $K_3$ in feedingstuffs, premixes and feed additives [6] | Sample extraction with chloroform, transfer vitamin K substances to free menadion; clean-up with Celite and sodium sulphate anhydrous; normal phase chromatography, Si-60 column 250 mm x 4 mm, 10 µm packing; UV detection at 251 nm | LOQ=0.5 mg/kg $RSD_r$ at 1 mg/kg: 10%; $SD_r$ at 8 mg/kg: 4%; $SD_r$ at the level >2500 mg/kg: 3%; |
| Vitamin A and E, feedingstuffs [7] | Sample hydrolyze with ethanolic KOH solution; clean-up on SPE column; elution in ethyl acetate, evaporation and dissolution in methanol; reverse phase chromatography, ODS2 column 250 mm x 4.6 mm, 5 µm packing; UV detection at 325 nm (vitamin A) and 292 nm (vitamin E). | Range: Vitamin A=1250-20000 U/kg; Vitamin E=3-300 mg/kg $RSD_{ip}$= 21% (vit. A), 11% vit. E; Rec: vit. A - 80%, vit. E - 110% |

**Table 1.** HPLC methods for the analysis of fat-soluble vitamins in feeds

| Analyte, matrix reference | Extraction, extract clean up and chromatography parameters | Performance Parameters |
|---|---|---|
| Vitamin $B_1$, $B_2$, $B_6$, NA, NSA in premixes and mineral feeds [8] | Extraction with methanol -Titriplex solution, clean-up on membrane filter 0.45 μm, reverse phase HPLC coupled to UV or diode array detector, column Nucleosil 250 x 3.0 mm, 5 μm packing; mobile phase: mixture of water solution of acetonitrile and acetic acid | Range, mg/kg: B1= 320-7940; B2= 868-15990; B6=627-11530; NA=4520-77850; NSA= 3665-61230; $RSD_r$(%) = 2.1-5.1; $RSD_R$(%) = 4.2-30.2 |
| Vitamin $B_1$ in feedingstuffs and premixes [9] | Extraction with methanol ; clean-up on SPE, reverse phase HPLC, coupled to a fluorescence detector, excitation at 360 nm, emission at 430 nm | LOQ=5 mg/kg; Range, mg/kg: 7 – 484; $RSD_r$(%)=4.2-4.7 $RSD_R$(%)=5-13 Rec.(%)=88-97 |

$B_1$ – thiamine; $B_2$ – riboflavin, $B_6$ – pirydoxin; NA- nicotin acid; NSA – nicotinamid;

**Table 2.** HPLC methods for the analysis of water-soluble vitamins in feeds

Numerous methods have been developed to examine coccidiostats in feedingstuffs with the use of high performance liquid chromatography. Examples of such methods are presented in Table 3. Satisfactory precision of such methods has been obtained, in conformity with that calculated from the Horwitz equation [3] and with the requirements of the Commission's Decision [19], which enables analyzing coccidiostats at the levels declared by manufacturers. Due to the hazard of cross-contamination with the remains of coccidiostats found in non-target mixed feeds on the production line and the risk of carry-over the remains of contamination onto the products of animal origin, it is necessary to continue lowering the limit of methods' quantification in order to control safe use of coccidiostats.

Table 4 presents HPLC methods for testing other feed additives, such as antioxidants, amino acids, methionine hydroxy analog. The official AOAC method for determining ethoxyquin was verified in testing pet food and meat meal [13]. Due to the determination of maximum content of antioxidants in feed mixtures for animals used for food production there is a necessity to check this method in testing typical feed mixtures and premixes. Amino acids are present in typical feed materials as components of proteins. In order to determine amino acids in feed materials it is necessary to subject proteins to hydrolysis and next to separate amino acids using ion-exchange chromatography and apply derivatization. With intensive animal production it is necessary to supplement the deficiency of amino acids, such as lysine, methionine, threonine and tryptophan. New feed additives have been registered recently, such as arginine, valine and cysteine. The official AOAC methods make it possible to determine mainly the composition and content of amino acids in feedingstuffs after hydrolysis [13], yet validation parameters of the determination methods have not been defined for all synthetic amino acids. Moreover, the precision parameters of the method used to determine amino acids with sodium metabisulphite or the hydrobromic acid method were in many cases unsatisfactory, which was confirmed by the values of the Horwitz ratio higher than 2, e.g. in a feed mixture for broiler chickens the HorRat (H) values

amounted to 1.7-3.6, with mean 2.5, while satisfactory H values are within the range of 0.5 > H > 2. This requires further studies with the use of high performance liquid chromatography in order to determine the total content of amino acids after hydrolysis and added amino acids.

| Analyte, matrix reference | Extraction, extract clean up and chromatography parameters | Performance Parameters |
|---|---|---|
| Halofuginone, medicated feeds [10,4] | Ethyl acetate extraction, purification by ion-exchange chromatography, reversed phase HPLC with UV detection at 243 nm, $C_{18}$ column (300 x 10 mm) 10 µm packing, mobile phase: mixture of acetonitrile and ammonium acetate buffer solution | LOQ = 1 mg/kg ; $RSD_r$ (%): 2.0-4.7; Rec.(%) 75.3-98.0; at the level of 3 mg/kg |
| Lasalocid, monensin, salinomycin and narasin, poultry feed [12] | Methanol extraction without clean-up, derivatization with 2,4-dinitrophenylhydrazide (DNP) in acid medium at 55 °C, ODS column (150 x 4.6 mm, 5 µm); eluent: methanol – 1.5% aqueous acetic acid (90:10, v/v), UV detection at 305/392 nm | LOQ = 40 mg/kg conc. range 50-150 mg/kg; $RSD_r$ (%): 4-10; Rec. 85-100% |
| Lasalocid, poultry feeds, premixes [11,4] | Extraction into acidified (HCl) methanol, agitation in ultrasonic bath at 40 ºC, filtration through a 0.45 µm filter, reversed phase HPLC, $C_{18}$ column (125 x 4 mm) 5 µm packing , mobile phase: mixture of phosphorus buffer solution and methanol 5+95 (v/v), fluorescence detector: excitation 310 nm; emission 419 nm | LOD=5 mg/kg; LOQ=10 mg/kg; $RSD_r$ (%): 2.1-5.4; $RSD_R$ (%): 5.0-10.7; Rec : feed ≥ 80%; premixes ≥ 90% |
| Robenidine, feedingstuffs, premixes [4] | Extraction into acidified (HCl) methanol, clean-up on an aluminum oxide column; reversed phase HPLC, UV detection at 317 nm; $C_{18}$ column (300 x 4 mm) 10 µm packing; mobile phase: mixture of acetonitrile and sodium and potassium phosphate solution | LOQ=5 mg/kg $SD_r$ (%): 3.3-5.4; $SD_R$ (%): 9.7-10.1; Rec. for blanc sample ≥ 85% |
| Diclazuril, feedingstuffs, premixes [4] | Extraction with acidified methanol with internal standard; purification on $C_{18}$ solid phase extraction cartridge (SPE), evaporation and dissolution in DMF; reversed phase gradient HPLC, Hypersil ODS column, 100 mm x 4.6 mm, 3 µm packing; mobile phase: (1) aqueous solution of ammonium acetate and tetrabutyl-ammonium hydrogen sulphate, (2) acetonitrile, (3) methanol | LOD=0.1 mg/kg; LOQ=0.5mg/kg; $SD_r$ (%): 1.9-17.3; $SD_R$ (%): 7.4-18.6; Rec. for blanc sample ≥ 80% |

$SD_r$ - standard deviation of repeatability; $SD_R$ - standard deviation of reproducibility; rec. – recovery; LOD – limit of determination; LOQ – limit of quantification; DMF – N,N-dimethylformamide;

**Table 3.** HPLC methods for the analysis of coccidiostats in feeds

| Analyte, matrix reference | Extraction, extract clean up and chromatography parameters | Performance Parameters |
|---|---|---|
| Ethoxyquin in pet-food and meat meal [13] | Extraction with acetonitrile without clean-up, reversed phase HPLC, $C_{18}$ column (250 x 4.6 mm) 5 µm packing , mobile phase: acetonitrile and 0.01 M ammonium acetate (70 + 30, v/v); fluorescence detector: excitation 360 nm; emission 432 | Method range: 0.5-300 mg/kg; $SD_r$ (%): 4.5-32; $SD_R$ (%): 4.5-55; Rec. 60-83% |
| Phenolic anti-oxidants* in fats [13] | Extraction with acetonitrile, extract is concentrated and diluted with 2-propanol; reversed phase gradient HPLC, $C_{18}$ column with guard column; mobile phase: (1) 5% acetic acid in water, (2) acetonitrile-methanol (1 +1, v/v) | Method range: 10-200 mg/kg; $SD_r$ (%): 2.1-11.5; $SD_R$ (%): 2.7-21.5; Rec. 83-103% |
| Tryptophan in feedingstuffs and premixes[4] | For total tryptophan alkaline hydrolise with saturated barium hydroxide solution; for free tryptophan extraction under mild acid conditions; reversed phase HPLC with fluorescence detector, excitation 280 nm, emission 356 nm; $C_{18}$ column (125 x 4 mm) 3 µm packing; mobile phase: acetic acid and 1,1,1-trichloro-2-methyl-2-propanol solution, pH 5.00 | Feedingstuffs: $SD_r$ (%): 1.6-1.9; $SD_R$ (%): 2.2-6.3; Feed materials: $SD_r$ (%): 0.8-1.3; $SD_R$ (%): 4.1-5.1; |
| Amino acids in feeds** [13] | Performic acid oxidation of the sample to oxidize cystine and methionine; amino acids liberation from protein by hydrolysis with 6 M HCl; dilution with sodium citrate buffer; amino acid separation on ion-exchange chromatograph with ninhydrin post-column derivatisation | Broiler feed: $SD_r$ (%): 1.1-4.7; $SD_R$ (%): 6.0-19.8; HorRat: 1.7-3.6 ~ 2.5 |
| MHA in feedingstaffs and premixes [8] | Extraction with water solution of acetonitrile; reversed phase HPLC with UV detection at 210 nm; RoSil-$NH_2$ column (250 mm x 4.6 mm, 5 µm packing) with guard column; mobile phase: acetonitrile with phosphoric acid solution ( 23+77) | LOD=0.2 g/kg LOQ=0.5 g/kg |

*PG-propyl gallate; THBT – 2,4,5-trihydroksybutyrophenone; TBHQ – *tetr*-butylhydroquinone; NDGA – nordihydroguaiaretic acid; Ionox 100 – 2,6-di-*tert*-butyl-4-hydroxymethylphenol BHA- 3-*tert*- butyl-4-hydroxyanisole; BHT – 3,5-di-*tert*-butyl—4-hydroxytoluene; OG, DG – octyl and dodecyl gallate

**Sodium metabisulphite method and hydrobromic acid method not applicable to determination of tyrosine and tryptophan; acid hydrolysis method not applicable for methionine, cysteine and tryptophan; MHA - methionine hydroxy analog

**Table 4.** HPLC methods for the analysis of other feed additives in feeds

## 3. A description of feed matrix and active substances in feed additives

The difficulty in determining certain feed additives is related with their low stability. In order to obtain a more durable form, resistant to the manufacturing conditions of feed mixtures, the additives are secured by protective coating. This concerns primarily vitamins A and $D_3$ , as well as feed colorants, such as canthaxanthin.

Vitamin A is produced in the form of gelatin-and-sugar beadlets or fat beadlets. Each beadlet contains ca. 0.5-0.6 μg of vitamin A, as calculated for retinal (ca. 2 IU). The distribution of beadlets in the feed is not equal and the feed enriched in vitamin A tends to segregate vitamin beadlets during the process of manufacturing and transporting the feedingstuff, especially in case of loose products. On the other hand, pelleting feed mixtures or subjecting them to other barothermal processes, such as extrusion or expanding reduces vitamin segregation, yet it lowers their durability at the same time. Ultimately, the unequal distribution of vitamins in feed may affect the precision and accuracy of results of analyses. Grinding the samples may improve the distribution of vitamin A, yet it will also increase the risk of its oxidation. Vitamin A is chemically unstable and its content and biological activity are reduced along with the presence of oxygen from the air, light, humidity, inorganic acids, choline hydrochloride, microelements and peroxides created in the processes of fat oxidation. It is recommended that samples should be ground immediately prior to an analysis into 1 mm particles. Further grinding of the sample before determining the content of vitamins may lead to their decomposition. A useful guideline regarding the preparation of samples for analyses, including the analyses of unstable feed additives such as vitamins, is provided by the currently issued ISO/FDIS International Standard 6498 [20].

Vitamins are protected against oxidation by antioxidants (e.g. ethoxyquin) which are added to the materials creating beadlets. Some forms which are physically and chemically stable are created in this way, e.g. the oleic form of vitamin $D_3$ and more stable compounds (menadione bisulfite, thiamine mononitrate and riboflavin phosphate). Feed additives used in feed manufacturing are introduced on mineral carriers or on wheat bran. The most frequently used mineral carrier is fodder chalk. In case of extracting additives from premixes with the use of diluted acids the influence of the carrier on the conditions of extraction should be considered. In such a situation it is recommended that the robustness of a method to slight changes in the analytical procedure or a change of matrix should be verified [2]. In analyzing feedingstuffs the interfering agent is fat which often occurs in significant amounts on feed mixtures (up to 10%). In high-fat samples containing more than 0.25g of fat in an analytical weight while determining fat-soluble vitamins, additional soaps are formed in the saponification process, which hinder the separation of the examined analyte.

It was possible to resolve the problem of interfering substances after using HPLC methods. However, the diversity of matrices and inhomogeneity of feedingstuffs pose numerous analytical problems while determining feed additives, such as vitamins or carotenoid colorants. The biggest difficulty is related to proper clean-up of the extract and selection of adequate conditions for chromatographic separation.

In case of vitamins, while examining the relevance of the producer's declaration and interpreting the result of the examination, one should be aware of the effect brought about by numerous factors on the content of vitamins in feedingstuffs. There were detailed studies in this respect conducted by Coelho [21]. Table 5, based on Coelho's article, presents the method of estimating the summary influence of different factors, such as the type of a premix and the time passed since the moment of its production (column 2), the type of conditions of hydro- and barothermal processing (column 3), feed storage time (column 4) on

vitamin retention. The product of the factors in columns 2, 3 and 4 (expressed as a fraction) will let us estimate the retention of a particular vitamin in a particular feed (column 5).

| Vitamin | Vitamin, premix (Coelho [21], Table 8) | Pelleting temp., (Coelho[21], Table 11) | Feed storage time (Coelho [21], Table 10) | Total vitamin reten. % |
|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 |
| | 2 months | 96 °C | 2 weeks | 2 x 3 x 4 |
| A beadlet | 90 | 88 | 98 | 78 |
| $D_3$ beadlet | 91 | 91 | 99 | 82 |
| E acetate 50% | 92 | 91 | 99 | 83 |
| Thiamine | 77 | 82 | 99 | 63 |
| Riboflavin | 91 | 84 | 99 | 76 |
| $B_{12}$ | 96 | 95 | 100 | 90 |
| Ca pantothenate | 87 | 84 | 99 | 72 |
| Biotin | 89 | 84 | 99 | 74 |
| Niacin | 90 | 86 | 99 | 77 |

**Table 5.** Vitamin stability in premixes and feeds (%), (Coelho, 2002)

Similarly, the authors observed in their own studies that the conditions and premix storage time in the laboratory affected the content of vitamins A and E. Fractioned samples of premix, each weighing 100 g, were stored for 8 months at room temperature (22 °C), in a fridge (5 °C) and in a freezer (-18 °C) (Table 6). In the samples stored at room temperature the content of vitamins after 8 months of storage was reduced to as little as 3% of the initial value. The analyses of vitamin content should be performed immediately after receiving the samples by the laboratory, otherwise the samples should be stored in a fridge until the analyses can be done.

| Item | Vitamin retention, %* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Month | | | | | | | | |
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Vitamin A, initial value 2794000 IU/kg: | | | | | | | | | |
| - room 22 °C | 100 | 94 | 82 | 64 | 32 | 13 | 6 | 4 | 3 |
| - refrigerator 5 °C | 100 | 102 | 99 | 94 | 94 | 93 | 93 | 92 | 85 |
| - freezer -18 °C | 100 | 100 | 100 | 100 | 99 | 100 | 98 | 99 | 96 |
| Vitamin E, initial value 15.56 g/kg: | | | | | | | | | |
| - room 22 °C | 100 | 98 | 96 | 94 | 94 | 97 | 95 | 94 | 90 |
| - refrigerator 5 °C | 100 | 99 | 98 | 100 | 98 | 100 | 100 | 98 | 90 |
| - freezer -18 °C | 100 | 99 | 97 | 100 | 99 | 100 | 100 | 100 | 94 |

* expanded uncertainty (k=2) of the result of examining is 16% for vitamin A and 12% for vitamin E

**Table 6.** The results of laboratory retention of vitamins A and E, %

## 4. Extraction and extract purification

A sample for a quantitative analysis should be prepared in such a way that the isolation of a selected analyte and removal of interfering substances is possible. The condition necessary for adequate quantitative determination in liquid chromatography is eliminating any possibility of coelution and minimization the drift of a basic line. Extractions of the analyzed substances were done classically by shaking the sample with a solvent. In case of vitamins $B_1$ and $B_2$ and carotenoids, such as canthaxanthin and apocarotenic acid ester, extraction was performed in an ultrasonic bath. In order to purify the extract, aluminum oxide (e.g. canthaxanthin), celite and anhydrous sodium sulphate (e.g. vitamin $K_3$) were used and PTFE and Nylon (PA) (0.45 μm or 0.20 μm) syringe filters were applied before injecting on the chromatographic column. Syringe (hydrophobic) PTFE filters are used in case of solutions with high acid and base content, whereas nylon (hydrophilic) filters are used with aqueous and organic solutions. Filtration of extracts is necessary as it prolongs durability of a column due to eliminating permanent contamination which blocks the column's intake and increases back pressure. If needed, the analyte may be concentrated by evaporating the solvent. When there is a risk that the studied analyte becomes oxidized, evaporation is done in neutral gas, e.g. nitrogen or argon.

The removal of gelatin-and-sugar beadlets protecting vitamins A and $D_3$ is done at the stage of saponification and transforming the vitamins into alcoholic forms. Douša & Břicháč [16] demonstrated that saponification in standard conditions did not affect the results of analyses. In case of canthaxanthin enzymatic hydrolysis through adding trypsin and pepsin is used. While determining the total content of thiamine and/or riboflavin in feedingstuffs (endogenic and added) at the stage of preliminary preparation an ultrasonic bath and also enzymatic hydrolysis (taka-diastase) are applied.

## 5. Optimization the conditions of chromatographic separation

The method of high performance liquid chromatography (HPLC) with flouorometric detection or a diode array is characterized by sufficient selectivity and sensitivity required to determine feed additives. Each of the developed and verified procedures includes precisely defined stages of hydrolysis and extraction which make it possible to determine the total or added analyte in a sample. Chromatographic separation of the analyzed mixture is affected by the properties of chromatographic arrangement. The developed analytical methods took advantage of chromatography in a regular and reversed arrangement of phases. In the reversed phase methanol and water or acetonitrile and water were used. In the standard arrangement the mobile phase was hexane or chloroform. In the majority of the developed methods presented in this chapter isocratic elution was applied, except for methionine hydroxy analog where gradient elution was used. Adequate separation was achieved through the use of ODS column with 18 atoms of carbon in the chain ($C_{18}$) and columns with 8 atoms of carbon ($C_8$) in the alkyl chain in chromatography with reversed phase (RP). In chromatography with the normal phase arrangement columns filled with silica gel were

used. The identification and content of analyte was examined with the method of absolute calibration (with external model), analyzing separately the sample and the model and identifying the peaks with the help of retention values, comparing retention time of the identified substance with the retention time of a standard, chromatographed in identical conditions. Examples of chromatograms for the standard extract and the sample of the examined analyte (riboflavin) with the use of fluorescent detector are presented below (Fig.1, Fig.2).

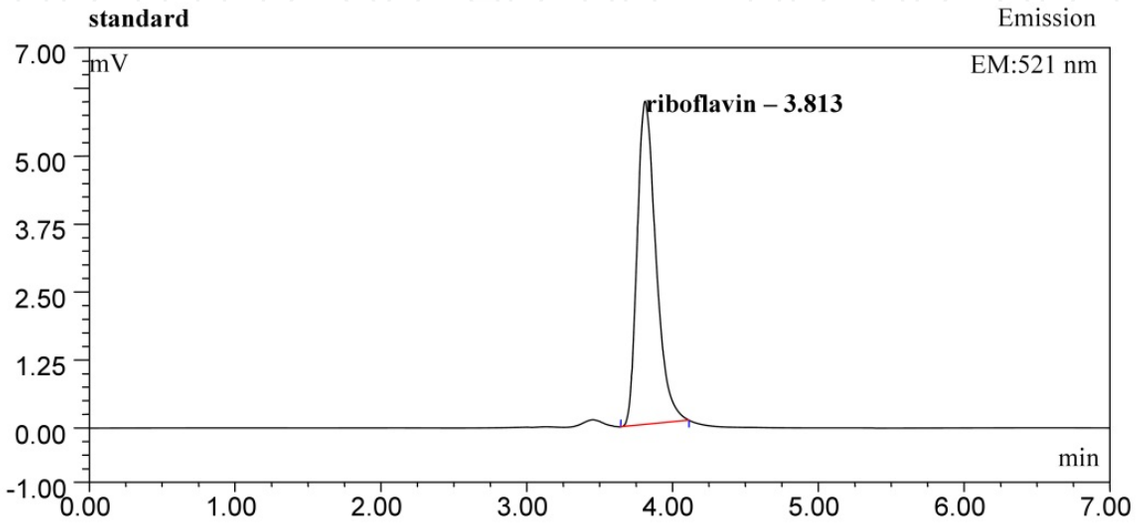



**Figure 1.** Characteristic chromatograms of riboflavin: chromatogram of standard solution

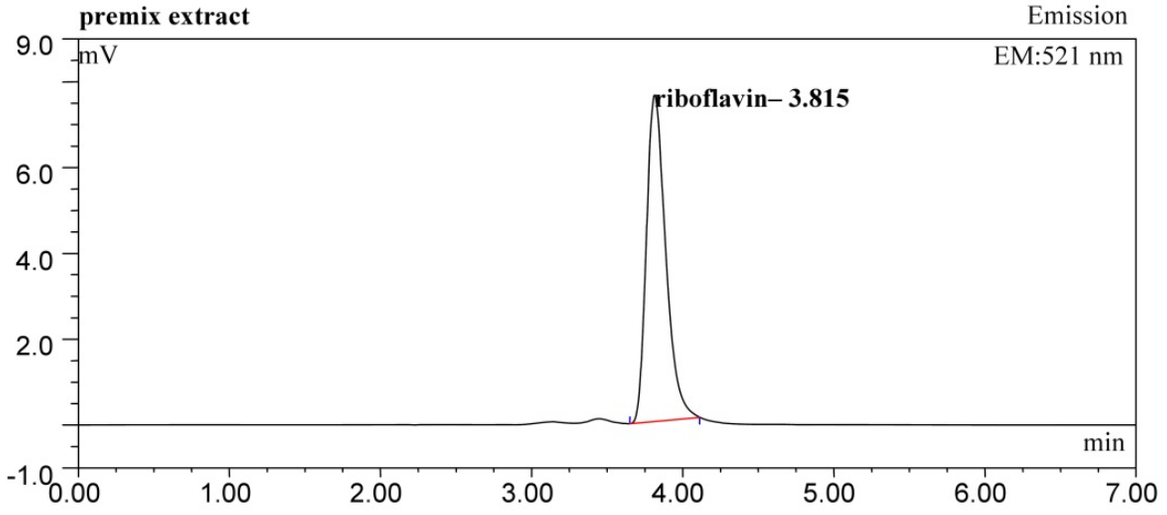



**Figure 2.** Characteristic chromatograms of riboflavin: chromatogram of premix extract

## 6. Selected methods of testing feed additives

Selected methods of testing feed additives presented below were validated with the help of a high pressure liquid chromatograph (Dionex P-680) with fluorescence detector (Dionex RF 2000) or with a diode array.

### Vitamin $B_1$ (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*. [18].

#### Principle

Vitamin $B_1$ 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.

**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 $B_1$ standard, taka-diastase, sodium acetate.

**Apparatus:** laboratory shaker, centrifuge, water bath with Allin condenser, HPLC set with fluorescence 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 $K_3Fe(CN)_6$.

#### Chromatography

| | |
|---|---|
| Column | 25 cm x 4.6 mm |
| Stationary phase | LichroCart 250-4, Lichrospher100 $NH_2$ (5μm) |
| Mobile phase | Chloroform and methanol, 90+10 (v/v) |
| Column temperature | 25 ºC |
| Flow rate | 2.0 ml/min |
| Injection | 20 μl |
| Detector | Fluorescence, Ex $\lambda$= 365, Em $\lambda$=435 |
| Calculation | External standard, peak area, linear regression |

#### Special Comment

This method was applied for the quantification of total content of thiamine in compound feedingstuffs as well as added thiamine in the form of hydrochloric or nitrate salt.

### Vitamin $B_2$ (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. [17].

#### 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

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 $B_2$ 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 |
| Stationary phase | $C_{18}$ |
| 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 $\lambda$= 453, Em $\lambda$=521 |
| Calculation | External standard, peak area, linear regression |

**Special Comment**

Vitamin $B_2$ 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. [22].

**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.

**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, activity 1; 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 |
| Stationary phase | LiChrospher Si 60 |
| Mobile phase | n-hexane: acetone 86:14 (v/v) |
| Column temperature | 25 ºC |
| Flow rate | 1.3 ml/min |
| Injection | 20 μl |
| Detector | DAD $\lambda$=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.* [25] and the official VDLUFA method [8].

**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.

**Chromatography**

| | |
|---|---|
| Column | 25 cm x 4.6 mm |
| Stationary phase | $C_{18}$, LiChrospher |
| 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, $\lambda$=214 nm |
| Calculation | External standard, peak area, linear regression |

**Special Comment**

- If the degree of MHA polymerization in the feed mixture is high and depolymerization in the conditions presented in the analytical procedure is incomplete it is necessary to increase the amount of hydroxide taken for hydrolysis and the amount of phosphoric acid for neutralization, keeping constant proportions, and next to take into account the change in the amount of the solution after hydrolysis, while calculating MHA content.
- The quality of separation on the chromatographic column depends on pH of the mobile phase. When acidity increases retention time for MHA is reduced. The excess of phosphoric acid in the injected solution after depolymerization reduces the time of MHA retention.

## 7. Validation parameters of the methods used to analyze feed additives

A significant element in verifying a chemical method, including chromatography, is its validation. Validation is a confirmation through examining and presenting some objective evidence that some particular requirements regarding the intended application have been fulfilled. The basic validation parameters include: calibration linearity, the limit of detection and quantitative determination, precision (repeatability, indirect precision, reproducibility), recovery and uncertainty.

Calibration linearity is defined as a relationship between the readings of the measuring device and the concentration of a particular component, in conformity with the regression equation: bx + a = y. The measure of linearity is Pearson's linear correlation coefficient (r) for parameters with regular distribution. The scale presented below is adopted to estimate the correlation coefficient: 0.0-0.2: very weak relationship; 0.2-0.4: weak relationship; 0.4-0.6: moderate relationship; 0.6-0.8: strong relationship; 0.8-1.0: very strong relationship.

In case of feed additives discussed in the present chapter, determined with HPLC methods, external calibration was used.

In case of chromatographic methods the value of limit of detection (LOD) may be determined on the basis of the obtained chromatogram of blanc sample, as the threefold value of a noise signal. To do this, it is necessary to determine the level of noise, by measuring on the chromatogram the range of signal change near retention time of examined

analyte. With chromatographic methods, the bottom limit of the method's application may be also regarded as the content of the analyzed component, which is equal to the lowest concentration of the standard used for calibration.

| Analyte | Matrix | LOQ | $CV_r$ % | $CV_{ip}$ % | Rec. % | Linear range |
|---|---|---|---|---|---|---|
| Vitamin A | Feedingstuff | 1000 IU/kg | 1.6 | 4.0 | 96.0 | 7.0-70 IU/ml; r=0.999 |
| | Premixture | | 1.4 | 2.0 | 95.2 | |
| Vitamin E | Feedingstuff | 6.0 mg/kg | 2.0 | 2.0 | 96.7 | 0.05-0.3 mg/ml; r=0.999 |
| | Premixture | | 1.0 | 2.0 | 96.4 | |
| Vitamin $K_3$ | Feedingstuff | 1.0 mg/kg | 6.4 | - | 100.9 | 0.046-4.62 μg/ml; r=0.999 |
| | Premixture | | 5.7 | - | 99.4 | |
| | Preparation | | 1.9 | - | 101.2 | |
| Vitamin $D_3$ | Premixture | 200 IU/g | 1.4 | 1.7 | 99.3 | 1.06-10.68 μg/ml; r=0.999 |
| | Preparation | | 1.3 | 1.3 | 98.4 | |
| Vitamin $B_1$ | Feedingstuff | 1.0 mg/kg | 5.6 | | 98.9 | 0.2-1.0 μg/ml; r=0.999 |
| | Premixture | | 3.7 | | 102.3 | |
| Vitamin $B_2$ | Feedingstuff | 1.0 mg/kg | 3.4 | 5.1 | 98.0 | 0.17-0.67 μg/ml; r=0.999 |
| | Premixture | | 2.3 | 6.2 | 98.3 | |

$CV_r$ – coefficient of variation; $CV_{ip}$ – intermediate precision; rec. - recovery

**Table 7.** Validation parameters obtained for selected feed additives – vitamins in feeds

| Analyte | Matrix | LOQ | $CV_r$ % | $CV_{ip}$ % | Rec. % | Linear range |
|---|---|---|---|---|---|---|
| Cantha-xanthin | Feedingstuff | 1.0 mg/kg | 4.7 | 7.9 | 97.3 | 0.7-8.5 μg/ml; r=0.999 |
| | Premixture | | 3.3 | 6.0 | 98.2 | |
| Tryptophan | Feedingstuff | 10.0 mg/kg | 4.1 | 4.0 | 94.9 | 12.5-100 nmol/l; r=0.999 |
| | Preparation | | 1.0 | 1.4 | 99.7 | |
| Ethoxyquin | Feedingstuff | 0.5 mg/kg | 2.0 | 6.0 | 99.0 | 0.01-0.07 μg/ml; r=0.999 |
| MHA | Feedingstuff | 0.05% | 2.8 | - | 96.7 | 0.05-0.45 mg/ml; r=0.997 |

**Table 8.** Validation parameters obtained for other feeds

During the validation process in a laboratory the precision of a method is determined through examining such parameters as repeatability and within-laboratory reproducibility (intermediate precision). Within-laboratory reproducibility may be calculated on the basis of control charts or from the range between parallel results of an analysis (replications) of a feed additive, in compliance with the Nordtest [23] handbook. For two or more replications for the analyses of an analyte in each sample it is necessary to calculate the mean value, the difference between measurements (range), relative difference in % and next mean relative difference (%) for all samples of a particular type of feed. The mean range divided by the

coefficient (for two replications d = 1.128) makes the standard deviation of within - laboratory reproducibility. In order to verify the method's precision the Horwitz ratio named HorRat (H), may be used which is the ratio of the relative standard deviation of reproducibility $SDR_t$ calculated from the Horwitz formula $SDR_t = 2\ C^{-0.15}$, where C stands for concentration expressed as a dimentionless mass fraction (e.g. 1 mg/kg = $10^{-6}$). In order to adjust it to the conditions of repeatability, target standard deviation $SDR_t$ is multiplied by 0.50 ($RSD_r = 0.5\ RSD_R$), [3]. Satisfactory values of the HorRat making the measurement of precision are included in the range of $0.5 < H < 2$ [3]. In case of participating in interlaboratory tests and obtaining satisfactory results, it is possible to include the precision parameters obtained in these analyses. The accuracy of a method may be determined by calculating the recovery degree or examining certified reference material, CRM.

## 8. Quality assurance and an uncertainty of result

Each laboratory should possess a program of quality assurance of its analyses within good laboratory practice. In case of chromatographic methods steering the quality may be implemented through performing one or more of the activities listed below:

- regular examinations of control samples;
- regular check-ups of the standard for each examination or series of analyses of labile feed components,
- checking b curve slope coefficient from the equation bx + a=y,
- analyzing overlapping samples (e.g. a solution of the sample for measurement, prepared and analyzed on the previous day),
- analyzing the blanc sample and fortified sample,
- examining certified reference materials, reference materials,
- participation in native and international proficiency testing.

Control material may be provided by certified reference material, CRM (matrix + analyzed substance), material from proficiency testing with a value assigned, enriched material prepared in the laboratory (fortified sample) and control material with recognized content of the tested and stable in time component, previously determined in the laboratory.

In compliance with the recommendations of the EN ISO/IEC 17025:2005 [24] standard and requirements defined in some regulations, in order to assess and interpret the result of a test, it is necessary to use the uncertainty of measurement. We hardly ever know the real content of the analyte and the result of the test is biased with an error. Hence, the idea of "uncertainty of measurement" has been introduced which is defined as "a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand" [26]. The EN ISO/IEC 17025:2005 standard recommends at point 5.4.6.2 that testing laboratories should possess and make use of procedures for assessing the uncertainty of measurement. To assess the uncertainty of methods used to analyze feeds the most frequently used approach is the model, consistent with the GUM [26] guidebook, which consists of finding the components of uncertainty and uses the law of error propagation. Using this particular approach to assess uncertainty, it is

possible to obtain an underestimated value in case when we do not consider all the components. Other reasons for underestimating uncertainty during validation include a situation when while assessing uncertainty repeatability instead of within-laboratory reproducibility is taken into account or we often forget to consider the bias. Moreover, uncertainty assessment is done during validation when well-ground typical samples are analyzed in a short time, new standards are prepared, the apparatus is controlled (standard conditions). During routine activities we analyze various matrices and obtaining homogeneity is not easy in case of some samples. The conditions mentioned above may affect underestimation of uncertainty. That is why it is important to have a possibility to verify uncertainty with the help of other approaches.

New opportunities concerning verification and assessment of uncertainty can be found in the Eurolab [27] technical report, the Nordtest [23] handbook and in the paper [28] which recommend the use of experimental approaches in assessing uncertainty of laboratory methods, in particular:

- within-laboratory experimental approach based on within -laboratory reproducibility and the assessment of the method's bias, following CRM,
- within -laboratory experimental approach based on within -laboratory reproducibility and the assessment of the method's bias, following participation in PT/ILC.

Using the within-laboratory experimental approach in assessing uncertainty of measurement (u) within -laboratory reproducibility (s) is considered as a measure of precision, as well as the bias (b), in accordance with the equation below following the Eurolab [27] technical report.

$$\sqrt{{s_{Rw}}^2 + b^2} = u \tag{1}$$

Precision of a research procedure in a laboratory is determined during validating the method or on the basis of measurements noted in control charts. Validation usually includes determining standard deviation of within-laboratory reproducibility $s_{rw}$ or standard deviation of within -laboratory reproducibility $s_{Rw}$ which is sometimes called intermediate precision. Bias is determined by means of standard deviation of measurement in relation to the reference value, e.g. while examining certified reference materials, using reference methods. The share of bias (b) in uncertainty of the measurement is determined with the help of mean deviation of measurements (Δ), uncertainty of reference material ($u_{ref}$ ) and precision of measurements during examining that bias (s). The value of the expression $s^2 / n$ with a bigger number of measurements is low and can be omitted:

$$\sqrt{\Delta^2 + u^2_{ref} + \frac{{s}^2}{n}} = b \tag{2}$$

In practice, different measurements result in different values of bias. In such a case the data may be combined and common assessment of a value of bias ($u_w$) may be determined as a function of the measured value or, for typical data of matrices and levels, according to the formula below:

$$\sqrt{\frac{\sum(bias_i)^2}{n}} = \Delta \quad (3)$$

When certified reference materials are lacking (a frequent situation) and when no other analyses of bias have been performed in the laboratory (e.g. prior to applying the reference method) bias can be estimated on the basis of proficiency testing, PT.

A laboratory participating in PT may use the results of such tests in order to assess uncertainty of measurement for the testing method/procedure used. Similarly to determining uncertainty in within-laboratory experimental approach, the uncertainty of measurement (u) is equal to the root of the sum of squared values of standard deviation for within-laboratory reproducibility $s_{Rw}$ and the bias (b), which can be calculated from the formulas 1,2 and 3.

With this approach two components of uncertainty are obtained from different sources. Precision is determined on the basis of the authors' own validation data (within-laboratory reproducibility), from the range or on the basis of measuring control charts (in-house). The bias is determined on the basis of PT results. Estimating the bias on the basis of a single participation in PT may have a limited range and should be treated as preliminary. If the data from several PTs are available (a wider range of matrices and concentrations) the assessment of the bias may be referred to the complete measurement range.

The results of analysing uncertainty on the basis of experimental approaches using the results of the authors' own results are presented below along with, for comparison, expanded uncertainties estimated with the help of Horwitz formula $RSD_R$ (%) = $2C^{-0.15}$ .

| Additive | Feed | $s_w$ (%) | bias (%) | u (%) | U = 2 ·u (%) | U (%) * |
|---|---|---|---|---|---|---|
| Vitamin A | Feedingstuffs | 4.0 | 12.4 | 13.1 | 26.2 | 23.8 |
| | Premixes | 2.0 | 7.2 | 7.5 | 15.0 | 11.8 |
| Vitamin E | Feedingstuffs | 1.0 | 9.0 | 9.1 | 18.2 | 16.1 |
| | Premixes | 2.0 | 6.1 | 6.4 | 12.8 | 8.2 |
| Vitamin $B_1$ | Feedingstuffs | 5.6 | 6.7 | 8.7 | 17.4 | 26.8 |
| | Premixes | 3.7 | 6.7 | 7.6 | 15.2 | 18.6 |
| Vitamin $B_2$ | Feedingstuffs | 6.52 | 3.16 | 7.2 | 14.4 | 24.0 |
| | Premixes | 5.09 | 3.16 | 6.0 | 12.0 | 10.7 |

*Expanded uncertainty for the HorRat value H=1 calculated from the Horwitz' formula $RSD_R=2\ C^{-0,15}$; U (%) = 2 $RSD_R$

**Table 9.** Results of uncertainty evaluation for some feed additives in compound feeds and premixes

## 9. Conclusion

The chapter presents a brief review of the methods used for determining feed additives by means of high proficiency liquid chromatography, HPLC. The authors presented their own research procedures and special attention was given to the preparation of samples for testing, extraction, extract purification, chromatographic separation and the basic elements of method validation and quality control.

Using HPLC for testing fat-soluble vitamins in feed materials, mixtures and premixes enabled us to replace colorimetric methods and to eliminate bias, such as the positive error of vitamin A determination related to the presence of carotenoids in the analyzed feed. The problem of low precision of examining certain vitamins, e.g. vitamin A, in feed mixtures is often unrelated to the method of determination, but rather to non-homogenous distribution of vitamin A in the feed related to its being secured against losing activity, due to protective coating. This problem may be solved by preparing the analytical weighed amount of sufficiently high mass and grinding the sample immediately prior to determination procedure to particles sized 1 mm.

Progress in the area of examining the content of water-soluble vitamins is also related to introducing the methods of liquid chromatography. The authors included their own procedures of analyzing vitamins $B_1$ and $B_2$, thiamine and riboflavin, with the use of HPLC methods and gave their characteristic parameters which meet the current requirements regarding the assessment of content and interpretation of results. These methods may be used especially to examine low content of thiamine and riboflavin, endogenic and added, in feed materials and mixtures.

HPLC methods have been widely used for testing cocciodiostats in feed preparations, premixes and mixtures. They contributed to improving the safety of using these additives, controlling concordance with manufacturer's declaration and not exceeding the maximum content in feed mixtures, as well as controlling the withdrawal period. Without liquid chromatography with mass spectrometry (LCMS) it wouldn't be possible to analyze effectively the remains of coccidiostats in the tissues and food products of animal origin. To reduce the risk of cross contamination in non-target feeds maximum content values for coccidiostats were determined recently at the level from 0.01 mg/kg (diclazuril) to 1.25 mg/kg (narasin, monensin), [29]. This created a need to develop some test methods adequate for the level of acceptable cross contamination and verifying them in interlaboratory tests. Future research will focus on checking the LCMS method for this particular purpose.

The official methods of separating and determining amino acids in feedingstuffs [13,8] are based mainly on ion exchange chromatography. However, in examining free amino acids (amino acids used as additives: lysine, methionine, threonine, tryptophan, valine, arginine and cysteine) HPLC methods are becoming increasingly more popular as they make the analyses shorter in time. In some cases a HCLP method is the only solution, e.g. while determining methionine hydroxy analog, verified in the authors' own studies. The need to perform a large number of analyses in a shorter time determines the direction of future studies of amino acids in feedingstuffs and using ultra-performance liquid chromatography, UPLC, for this purpose.

In the testings of feed colorants the most frequently used means were spectrophotometric methods [13,8]. The diversity of feed products and the resulting changeability of matrix, as well as determining the maximum content of colorants in feed mixtures, were the reasons for searching for new methods of examining colorants, including HPLC. An example of such a method in reference to canthaxanthin and a procedure based on the authors' own research is quoted in the present work. Future research in this respect will use the LCMS method to a higher degree as it enables detecting and determining several feed colorants in a single sample in view of cis-trans steroisomers.

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