ACTA UNIVERSITATIS PALACKIANAE OLOMUCENSIS
FACULTAS RERUM NATURALIUM (2010) CHEMICA 47

HPLC determination of vitamin A in the animal feeds – the sources of errors and their reduction

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Abstract
This study provides a comprehensive review on the determination of vitamin A in the animal feedstuffs. There are many methods for determination of vitamin A in the feeds. For regulatory purposes, the relevant European Commission in European Union specifies the methods which are applicable and suitable. However, some feed analysts and some national organizations continue with their own vitamin A methods. If possible, a single, widely applicable general method should be used for vitamin A in animal feeds. Vitamin A may be determined by spectrophotometric and fluorometric procedures. Sometimes, chromatography is required as an important part of the method. The above described situation produces many errors in analytical measurements and results. This study describes some source of possible error in vitamin A analyses.

Keywords: HPLC, vitamin A, interlaboratory study

1. Introduction

The determination of vitamin A in the animal feeds includes several consecutive steps: sample preparation, alkaline hydrolysis of sample, extraction of vitamin A from saponified oils, clean-up of extract and HPLC separation. Vitamin A is chemically unstable and its biological activity decreases in the presence of hydroperoxides, air, light, moisture, mineral acids, heavy metals and additional oxidants. All operations with vitamin A solutions and vitamin-containing materials should be carried out in subdued light and as far as possible in low actinic amber glassware. All the above-mentioned factors are the reason of high value of interlaboratory standard deviation of vitamin A determination.

2. The sources of errors in vitamin A analysis

2.1 Sample handling and preparation

The samples of animal feeds containing vitamin A should be protected from direct sunlight, excessive heat and moisture during transport, storing and sample preparation in the laboratory. Regarding its instability vitamin A is added in form of gelatin or fat capsules, each containing about some amount of vitamin A. Distribution of the capsules in feeds is not uniform in respect to size and number of capsules hence the fortified feed has the tendency to separate or agglomerate during handling and sample preparation. Grinding of samples could improve the distribution of vitamin A.

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On the other hand, risk of vitamin A oxidation has to be taken into account. The final consequence is that distribution can affect the validity of analytical results. Lehman [1] calculated the minimum sample size needed for vitamin A analysis regarding the size of particles and distribution of vitamin A in feeds (the prediction of the fact that the particle are not of uniform size or vitamin A content). The minimal theoretical relative standard deviation (RSD) of vitamin A analysis for various feeds was calculated to be 13 – 30 % when a 40 g sample was used for an analysis and error of respective analytical methods is negligible. Later Bourgeois calculated that the RSD increased from 10 to 30 % when weight of sample using for vitamin A analysis decreased from 100 to 10 g [2]. To obtain satisfactory results the sample weight could not represent less than 100 vitamin A-containing capsules for a same concentration of vitamin A.

It can be concluded that sample preparation depends on the weight of sample, vitamin A content, feed matrix and distribution of particles of vitamin A. In face of these presented facts the sample procedure has to be optimized – for example the size of aggregated sample would be greater than the usual. The analytical method should not be miniaturized by using smaller sample weight and reagent volumes. The initial sample weight should be as large as possible and the next steps of sample preparation (grinding, pounding, mixing) must be realized immediately before the analysis (especially mineral premixes, high-fat feeds and pet foods) [3].

2.2 Saponification

The extraction of vitamin A from feeds is routinely performed by saponification. The vitamin A is not extracted quantitatively from fortified feed containing protected vitamin A with simple extraction by organic solvent. The coating must be firstly penetrated by treatment with alkali and this is one of the reasons for the use of alkaline hydrolysis in the analysis of feeds [4]. The alkaline hydrolysis has other useful consequence for the release of vitamin from gelatin capsules. All vitamin A esters are converted to the vitamin A alcohol (retinol), fats (lipids and phospholipids) are converted to salt of fatty acids (soaps) and glycerol and a large number of nature pigments, antioxidants and others substances are converted to water-soluble compounds.

The alkaline hydrolysis is carried out by methanolic or ethanolic KOH in presence of antioxidants under reflux in a special hydrolysis apparatus at 70 °C with operating time of 30 minutes. Glycerol can be added to all samples as a solubilization aid for gelatin capsules. Use of glycerol is important if methanol is used instead of ethanol, because methanol is not as suitable solvent for fats as ethanol. The amount of alcoholic KOH solution required is dependent on the fat content in sample. A rough guide is to use 5 ml of 60 % aqueous KOH and 15 ml of alcohol per 1 g of fat in the sample. A slow stream of nitrogen into the saponification flask is introduced during hydrolysis hence the saponification medium is saturated with nitrogen. Thus, the present vitamins are protected from aerial oxidation during boiling. Vitamin A is stable in presence of alkali and conversely unstable in presence of acids, which are promoters of its *cis-trans* isomerization. Retinol acetate and retinol palmitate are more resistant to oxidation than free alcohol [5]. The degree of isomerization depends on the type of feed and on the conditions of saponification (the isomerization is catalyzed by mineral substances and enhanced temperature – thermal isomerization) and ranges from 4 to 40 % [6]. This problem can be solved by cold saponification (overnight) at ambient temperature [7]. The important step of hot saponification is end of hydrolysis and fast cooling of the reactive

medium to room temperature under a stream of cold water or in ice bath. The usual antioxidants are used in prevention of vitamin A degradation during saponification. Pyrogallol [8], ascorbic acid, butylhydroxytoluene [9] has been reported as suitable antioxidants. However, some antioxidants (e.g. butylhydroxytoluene or pyrogallol) are extracted into organic phase [10] and this may cause an increase of background fluorescence during detection of vitamin A.

2.3 Extraction and clean-up procedures

The extraction using organic solvent is one of the largest sources of errors in vitamin A analysis. The carotenoids, fat-soluble vitamins, sterols, and the other compounds are extractable from the saponification digest by liquid-liquid extraction using water-immiscible organics solvents. The fatty acids presented as their salts and glycerol are not extractable under alkaline conditions. Recently, extraction of vitamin A using various hydrocarbon organic solvents, e.g. diethyl ether [11], petrol ether [12] or hexane [13] was reported. Vitamin A is readily extracted by hexane from aqueous ethanol. The ethanol-water-soap mixture in the digest tends to behave similarly to a hydrocarbon solvent, decreasing the affinity of the fat-soluble vitamins to the organics phase (distribution constant is low). To maintain the high recovery of vitamin A it is important to optimize ratio of water and ethanol and concentration of fatty acids in the extraction system [14]. The concentration of ethanol is ranging from 30 to 40 % [14]. The dependence of vitamin A extraction yield to hexane versus content of ethanol in extraction system is showed in Fig. 1.

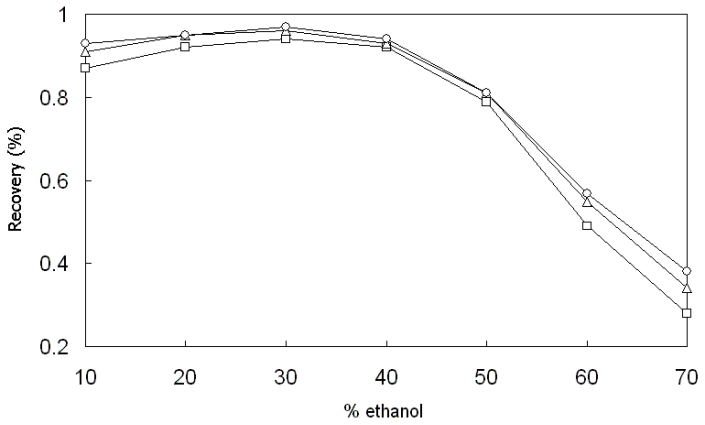

Fig. 1: Dependence of recovery of etraction of vitamin A to hexane on content of ethanol in extraction system. Concentration of vitamin A: □- 2800 IU l^{-1}; Δ - 7300 IU l; o – 15000 IU l^{-1}.

The efficiency of extraction using hexane is dependent on the concentrations of fatty acids in the digest. The recovery of vitamin A decreases with increasing levels of fat. In high–fat samples (more than 0.25 g per sample weight), extra soaps, formed during saponification, will change the distribution constant in favour of the aqueous-alcohol phase. Than the increase of the number of extraction steps is necessary to enhance of recovery of vitamin A. The influence of content of fat in saponification digest on yield of extraction of vitamin A is showed in Fig. 2 [15]. Control is achieved by limiting the amount of fat in the sample, by optimizing the amount of water added before extraction, and by repeated extractions with small volumes of hexane. In the presence of soaps, water and hydrophobic

solvents, the stabile emulsion is produced by shaking in the absence of ethanol. The growth of emulsion is limited by addition of neutral salts (e.g. sodium chloride).

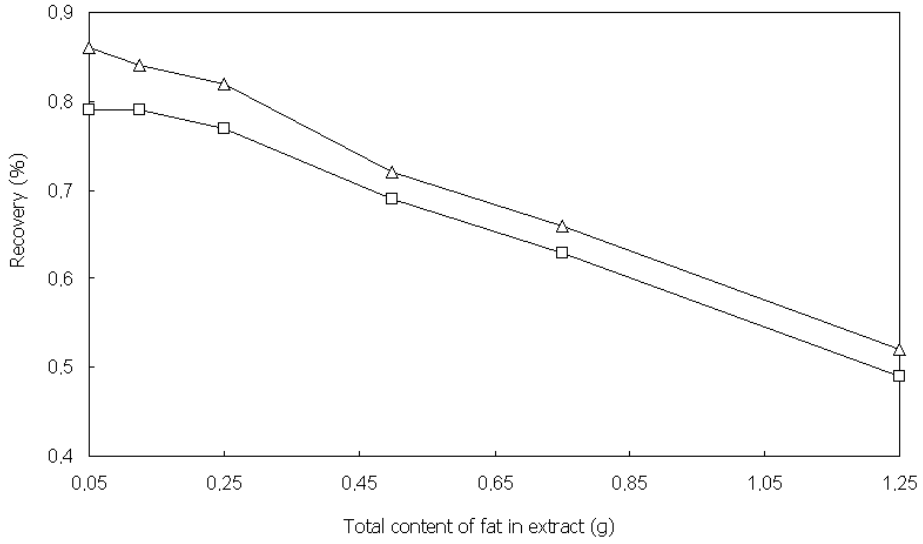

Fig. 2: Influence of content of fat in saponification digest on recovery of extraction of vitamin A. Concentration of vitamin A: □ - 2700 IU l^{-1}; Δ - 14500 IU l^{-1}.

The organic extract of vitamin A is dried by using the filtration through the layer of anhydrous sodium sulfate and then the sample is evaporated to dryness under vacuum. During solvent evaporation, vitamin A might be degraded if too high temperature is used. The temperature should be kept below 40 °C. The residue must not be exposed to air. The last residue of solvent should be evaporated under the stream of nitrogen or should be dissolved in appropriate solvent immediately upon drying.

The intensity of shaking during extraction by hexane is critical for complete recovery. It is necessary to optimize the shaking time, the minimum number of extraction steps (it is better to use 4 consecutive extractions), the volume of the extraction solvent and the extraction technique.

Clean-up procedures include open column chromatography, solid phase extraction, liquid-liquid extraction, thin-layer chromatography, and high-pressure gel permeation chromatography. The more recent applications of open column chromatography utilize liquid-solid chromatography using gravity flow glass columns dry-packed with magnesia, alumina, or silica gel.

The technique of Extrelut used to determine content of vitamin A and vitamin E in animal feeds was described in 1985 [16]. This technique was later modified for high-fat samples [17]. Disadvantage of this method is that the mechanism of separation is not exactly described and the composition of used adsorbent is not always representative.
Recently, the clean-up procedures are not used if it is not required or necessary.

2.4 Chromatographic separation and detection

The advantage of HPLC method is separation of vitamin A isomers from the other components of matrix which are extracted to non-polar solvents. Therefore, it is not necessary the clean-up of crude extract. The normal and reverse phases can be used for the separation

of vitamin A. The analysis using normal phase (silica) allows the separation of six *cis*- *trans* – isomers of vitamin A (11,13-di-*cis*-, 13-*cis*-, 11-*cis*-, 9,13-di-*cis*, 9-*cis* and all-*trans*-retinol) using mobile phase hexane or cyclohexane with addition of 2-propanol or ethanol as a modifier [18]. For the other modification of mobile phase have been used dichloromethane, isopropyl ether and chloroform [19]. The glycerides and fatty acids are retained on silica too strongly and to clean up the column it is recommended to wash it with 25 % ethyl acetate in hexane [20].

The most frequently used reverse phase for separation of vitamin A is octadecylesilane (C_{18}) with variously modified mobile phases - methanol + water and acetonitrile + water. The separation on reverse phase has specific restriction – the *cis-trans* isomers are not separated and the total amount of all isomers of vitamin A is determined only. Analytical Methods Committee decided that it would be preferable to select the conditions for the chromatography such that all of the isomers of the vitamin A were eluted in one peak. The results of interlaboratory studies have already been released [21]. The analysis of vitamin A was carried out on the reverse phase C_{18} with mobile phase methanol + water (97+3). The interlaboratory coefficient of variation was calculated ranging from 10 to 30 % (for content of vitamin A from 6 000 IU kg^{-1} to 48 000 IU kg^{-1}). Advantages of reverse phase systems to normal-phase chromatography include following : less sensitivity to changes in retention time due to presence of water; more stable to small changes in mobile phase composition; fast equilibration after mobile phase composition changes, permitting use of gradients and capability of resolving compounds with a wide range of polarities.

The spectrophotometric, fluorimetric or electrochemical detector can be used for the detection of vitamin A. The absorbance maximum of retinol and its esters is 325 nm and limit of detection is about 2 ng [22]. The majority of lipids have absorbance maximum below 220 nm, conjugated fatty acids about 230-235 nm (dienes) or 260-280 nm (trienes) and do not interfere [23]. Glycerides and sterols show maximum absorbance at 265 – 266 nm. Fluorescence can be used for detection of retinol and retinyl esters. However, oxidation of the alcohol essentially quenches the fluorescence. Fluorescence excitation spectrums of retinol and its isomers correspond to absorbance spectrums and maximum is ranging from 324 to 328 nm; emission maximum is ranging from 470 to 490 nm and detection limit is about 0.5 ng (on column) [24]. The electrochemical detection is less selective than the other ways of detections, the detection limits are comparable with fluorescence detection [25]. Although the use of electrochemical detection is advantageous in cases where sample amounts are highly limited, most routine assays do not require such low detection limits, and UV detection is more than sufficient.

2.5 Standards and determination of vitamin A concentration in the standard solutions

The improper calibration can be source of errors in the determination of vitamin A in feeds. It is impossible to obtain accurate analytical results if the reliable purity of external standard is not known accurately. The content of vitamin A in commercial standard (retinyl acetate or retinyl palmitate) is ranging from 50 to 140 % of its declared value [3]. Even when the original standard is of excellent purity, it may deteriorate as a result of improper packaging. Consequently rigorous concentration of standard solution of vitamin A must be determined after its alkali hydrolysis and extraction to organic solvent using spectrophotometric method with absorbance maximum from 324 to 327 nm.

The concentration of standard [IU g^{-1}] of vitamin A is calculated from following equation:

$$c_A = 10^7 . \frac{A.V.R}{0{,}3.A_{1cm}^{1\%}.m}$$

where A is absorbance of vitamin A standard solution (optical path length 1 cm), V is volume of extract in organic solvent (liter), m is mass of standard of vitamin A (gram) and R is dilution. The values of $A_{1cm}^{1\%}$ are tabulated (Table 1 [26,27]). The proper value of $A_{1cm}^{1\%}$ dependents on organic solvent and it is recommended to measure the absorbance in isopropanol where $A_{1cm}^{1\%}$ = 1827. This is the procedure used in pharmacopeia assays and it is recommended in food analysis.

Table 1: Tabulated value of for vitamin A

Form of vitamin A	**Solvent**	λ_{max} (nm)	$A_{1cm}^{1\%}$
all-*trans* retinol	ethanol	324-325	1832-1835
	2-propanol	325,5	1821-1835
	cyklohexane	326-327	1735
all-*trans* retinolacetate	ethanol	325-326	1560
	2-propanol	324-326	1530-1560
	cyklohexane	327-328	1520
all-*trans* retinolpalmitate	ethanol	328	940
	2-propanol	326	957

3. Experimental part

The described method to determine the content of vitamin A in feeds was used [21], but the extraction of vitamin A was carried out with hexane instead of light petroleum. In this study, the following parameters were studied:

a) the influence of sample weight on RSD in one laboratory
b) the influence of standard of vitamin A on mean and RSD in the interlaboratory study
c) the correlation between cold and hot saponification

The method for determination of vitamin A content in complete feeds using cold saponification was modified by increasing the amount of sample weight from 50 to 80 g (simultaneously the volume of ethanolic potassium hydroxide solution is increasing in the same ratio as in the original method). The saponification was carried out over night on rotation shaker at ambient temperature. The aliquot part of saponified digest was pipette to perform extraction by hexane. The rest of the procedure was the same as in the original method.

Three samples of premixes with declared content of vitamin A: 1 000 000 IU kg^{-1} (sample 1), 3 000 000 IU kg^{-1} (sample 2) and 2 200 000 IU kg^{-1} (sample 3) were circulated for the interlaboratory study. The results obtained in the interlaboratory study are evaluated for two

standards of vitamin A. The laboratories used their own standard ss the first standard and the second standard (Sigma-Aldrich, USA, approximated value 250 000 UI g^{-1}, determined value 323 000 UI g^{-1}) was distributed to the participant laboratories simultaneously with the samples. The step of saponification was carried out with two different amounts of sample weights – 5 g and 15 g.

4. Results and discussion

It was found that the amount of sample weight had significant influence on RSD. The RSD for content of vitamin A from 20 000 to 40 000 IU kg^{-1} was calculated and it was ranging from 12 % to 18 % when an 80 g sample was analysed. The RSD increases to 24 - 30 % when 15 g of feed was used. The conclusion is the most evident for lower content of vitamin A (Fig. 3): the amount of sample weight considerably affected the RSD.

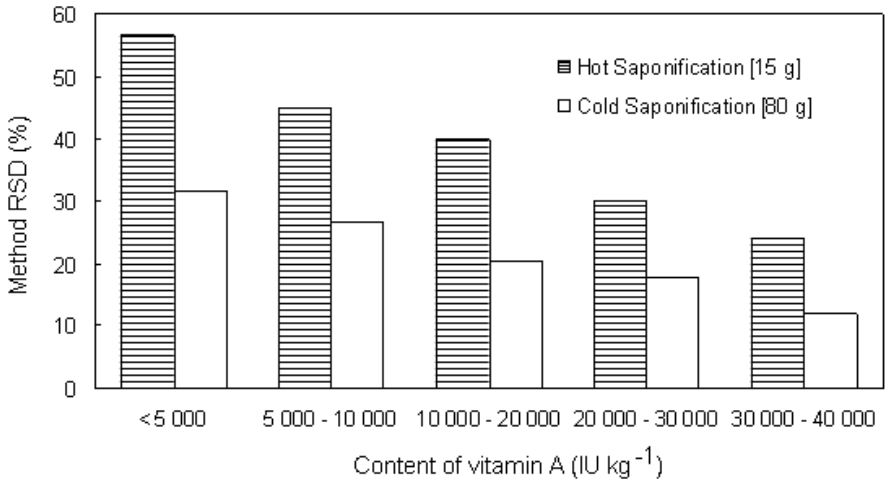

Fig. 3: Influence of hot and cold saponification on relative standard deviation of determination of vitamin A.

The results obtained in interlaboratory study are summarized in Table 2 – 4. Despite all expectations, the interlaboratory RSD for content of vitamin A from 1 000 000 to 3 000 000 IU kg^{-1} slightly decrease with increasing amount of sample weight. The interlaboratory RSD is ranging from 8.8 % to 10.5 %. On the other hand, the standard of vitamin A used for calibration had significant influence on interlaboratory RSD. When the circulated standard of vitamin A for calculation of content of vitamin A was used the interlaboratory RSD decreased by approximately by 50 % for sample 1 and 2 and it is ranging from 4.4 to 5.2 %.

The cold and hot saponification were tested to verify the temperature influence during saponification. The test was carried out on the same samples of complete feeds and for the same amount of sample weight. The number of samples used for the test was 28 and the content of vitamin A was ranging from 3 000 IU kg^{-1} to 32 000 IU kg^{-1}. Determined values were compared using linear regression. The regression equation (significance level P=0.95) was y = 33.261 + 1.0285 x (r =0.9035). The temperature slightly affected the amount of vitamin A and it can be concluded that temperature of saponification has not a significant influence on the amount of vitamin A (Fig. 4).

Table 2: The results of interlaboratory study. Determination of vitamin A in premix with HPLC – sample 1.

Laboratory	Sample weight		
	5 g	**15 g**	**15 g** [a]
2	1 160 730	1 027 358	1 097 943
5	1 104 302	1 043 326	1 041 678
7	998 679	1 073 321	1 079 626
8	1 110 206	1 056 634	1 043 287
9	1 110 500	1 110 000	1 115 000
10	885 926	881 615	991 275
11	851 000	845 000	845 000 *)
12	718 102 *)	1 010 911	1 013 560
14	1 233 369	1 293 567 *)	1 166 771 *)
15	1 053 200	992 950	994 350
16	866 329	874 164	872 343 *)
17	1 053 000	970 000	1 002 000
Means [IU kg^{-1}]	1 038 840	989 571	1 042 080
Interlaboratory standard deviation [IU kg^{-1}]	125 562	87 782	46 272
Relative interlaboratory standard deviation [%]	12.1	8.9	4.4

Annotation:
a – the results obtained for the circulated standard of vitamin A

*) – the results were excluded from this statistics

Table 3: The results of interlaboratory study. Determination of vitamin A in premix with HPLC – sample 2.

Laboratory	Sample weight		
	5 g	**15 g**	**15 g** [a]
2	2 323 111	2 549 500	2 723 880
5	1 812 563	2 206 866	2 203 013
7	2 247 514	2 190 448	2 244 650
8	2 365 831	2 511 919	2 479 125
9	2 500 000	2 310 000	2 400 000
10	2 184 200	2 235 663	2 188 280
11	1 923 000	1 916 000	1 916 000
12	2 029 330	2 326 774	2 331 918
14	2 688 074	1 854 635	2 004 196
15	2 267 850	2 135 150	2 138 150
16	2 653 711	2 636 663	2 588 983
17	2 440 000	2 430 000	2 640 000
Means [IU kg^{-1}]	2 286 265	2 275 302	2 321 516
Interlaboratory standard deviation [IU kg^{-1}]	271 042	239 069	252 701
Relative interlaboratory standard deviation [%]	11.9	10.5	10.9

Annotation:
a – the results obtained for the circulated standard of vitamin A

*) – the results were excluded from this statistics

Table 4: The results of interlaboratory study. Determination of vitamin A in premix with HPLC – sample 3.

Laboratory	Sample weight		
	5 g	**15 g**	**15 g** [a]
2	2 323 111	2 549 500	2 723 880
5	1 812 563	2 206 866	2 203 013
7	2 247 514	2 190 448	2 244 650
8	2 365 831	2 511 919	2 479 125
9	2 500 000	2 310 000	2 400 000
10	2 184 200	2 235 663	2 188 280
11	1 923 000	1 916 000	1 916 000
12	2 029 330	2 326 774	2 331 918
14	2 688 074	1 854 635	2 004 196
15	2 267 850	2 135 150	2 138 150
16	2 653 711	2 636 663	2 588 983
17	2 440 000	2 430 000	2 640 000
Means [IU kg^{-1}]	2 286 265	2 275 302	2 321 516
Interlaboratory standard deviation [IU kg^{-1}]	271 042	239 069	252 701
Relative interlaboratory standard deviation [%]	11,9	10,5	10,9

Annotation:
a – the results obtained for the circulated standard of vitamin A

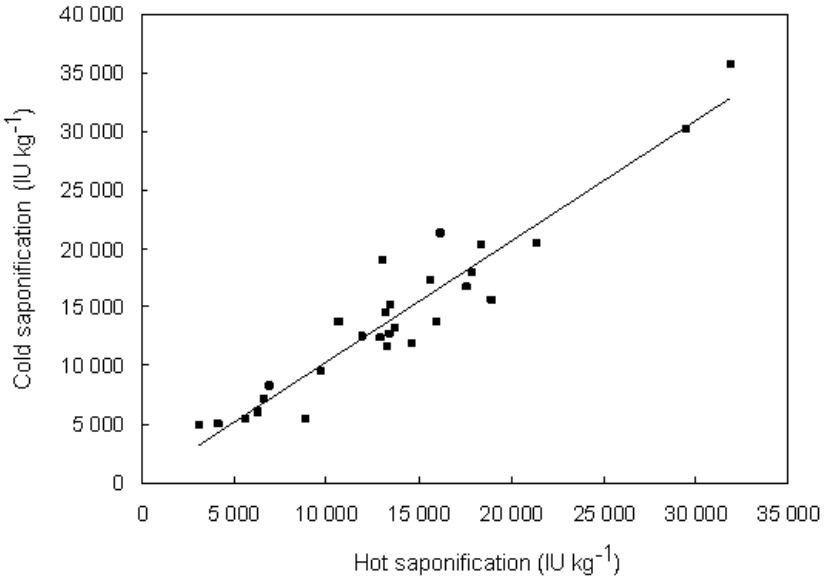

Fig. 4: Comparison of results of cold with hot saponification.

5. Conclusion

Errors in analytical measurements are generally of three types: human errors, errors due to bias (called systematic positive or negative errors), and errors due to random causes. The same conclusions could be applied to any potential sources of errors in analysis of vitamin A in animal feeds.

Improper using a standard of vitamin A is one of source of error. The systematic errors producing a positive bias are following: use of degraded standard, failure to remove standard solution from the evaporator immediately after hexane removal and also poor recovery of the standard at the partitioning step. On the other hand, the higher actual standard concentration of vitamin A caused a negative bias. The concentration of standard solution by spectrophotometric method must be checked carefully and it is necessary step. Also confirmation (wavelength and absorbance) of spectrometers used for determining standard concentration is essential.

The poor precision of described method is mainly caused by using of small sample size. To avoid poor precision the application of minimally a 15 g of sample weight for premixes and an 80 g of sample weight for final feeds is necessary.

The influence of temperature during saponification has not been observed.

5. References

[1] R.W. Lehman, J. Assoc. Off. Agric. Chem. 43 (1960) 15-20.
[2] C.F. Bourgeois, Analysis 12 (1984) 84-89.
[3] N. Thiex, R. Smallidge, R. Beine, J. Assoc. Off. Anal. Chem. 79 (1996) 1269-1274.
[4] M.L. Coley, J. Agric. Food Chem. 37 (1954) 742-744.
[5] W.J. Dann, Biochem. J. 26 (1932) 666-678.
[6] H. Steuerle, Z. Lebensmittel.- Unters.-Forsch. 181 (1985) 400-403.

[7] J.N. Thompson, W.B. Maxwell, M.L. Abbe, J. Assoc. Off. Anal. Chem. 60 (1977) 998 -1009.
[8] J.W. DeVries, D.C. Egberg, J.C. Heroff (1979) in book Liquid Chromatographic Analysis of Food and Beverages, page 477-497, 2nd volume, Academic Press, New York.
[9] VDLUFA - Verband Deutscher Landwirtschaftlicher Untersuchungs und Forschungsanstalten (1988): 2nd volume, part 13.1.2., Methodenbuch Band III.
[10] S.L. Taylor, M.P. Lamden, A.L. Tappel, Lipids 11 (1976) 530-539.
[11] J.N. Thompson, G. Hatina, J.Liq. Chromatogr. 2 (1979) 237-241.
[12] S.L. Reynolds, H.J. Judd, Analyst 109 (1984) 489-492.
[13] H.E. Indyk, Analyst 113 (1988) 1217 - 1223.
[14] J.N. Thompson J. Assoc. Off. Anal. Chem. 69 (1986) 728 -738.
[15] M. Douša, (1997) in Bulletin of ´97 laboratory department (in Czech), volume I, part 1/1997, ÚKZÚZ Brno, Czech republic.
[16] C.F. Bourgeois, J. Assoc. Off. Anal. Chem. 68 (1985) 1121-1130.
[17] C.F. Bourgeois, J. Assoc. Off. Anal. Chem. 71 (1988) 12-18.
[18] B. Stancher, F. Zonta, J. Chromatogr. 287 (1984) 353-359.
[19] G.M. Landers, J.A. Olson, J. Chromatogr. 291 (1984) 51-57.
[20] J.N. Thompson, G. Hatina, W.B. Maxwell, J. Assoc. Off. Anal. Chem. 63 (1980) 894 -898.
[21] Analytical Methods Committee, Analyst 110 (1985) 1019-1026.
[22] P.V. Bhat, P.R. Sunderesan, CRC Crit. Rev. Anal. Chem. 20 (1988) 197-204.
[23] F.D. Gunstone, F.A. Norris (1983) in book: Lipids in Foods. Chemistry, Biochemistry and Technology, Pergamon Press, Oxford.
[24] C.A. Collins, C.K. Chow, J. Chromatogr. 317 (1984) 349-355.
[25] W.A. MacCrehan, E. Schönberger, Clin. Chem. 33 (1987) 1585 - 1591.
[26] J.T. Gillingham, J. Assoc. Off. Anal. Chem. 50 (1967) 828-833.
[27] D. Glick, J. Biol. Chem. 156 (1944) 643-647.