Central Institute for Supervising and Testing in Agriculture
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STAATLICHE BETRIEBS-GESELLSCHAFT FÜR UMWELT UND LANDWIRTSCHAFT

Freistaat SACHSEN

Report

Validation study of the determination of vitamin A, E and D content - Method using solid phase extraction clean-up and High-Performance Liquid Chromatography

Report on the WI 00327115

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1 Background

The mandate M/523 issued by the European Commission in the framework of the Regulation (EC 882/2004) on official controls includes in third part standardization of method for determination of vitamin A, E and D_3.

The establishment of standardized methods of analysis is of utmost importance to guarantee a harmonised application and control of the European legislation in all Member States and to guarantee a high degree of feed and food safety.

The mandate M/523 was sign by all responsible parties on January 2016.

2 Introduction

Vitamins are essential organic compounds, that are necessary for normal body functions. Adding vitamins to feeds should lead to higher feed conversion, better productivity and improvement of animal welfare.

Single method for determination of fat-soluble vitamins (A, E) were formerly standardized by ISO and CEN. This newly proposed procedure improves existing method by analysing an additional vitamin (D_3) and use a rapid cleaning procedure (SPE) which saves time and chemicals.

Furthermore, this method is a multi-method which makes possible simultaneous determination of three vitamins – A, E and D_3.

The present project was performed in the framework of the Third Mandate M/523-2 of the CEN/TC 327 to standardize the method for simultaneous determination vitamin A, E and D_3 in animal feed by HPLC/UV or FLD.

3 Scope

A validation study is mandatory to standardize a method in the frame of CEN/TC 327. A collaborative study will be organized to validate the draft method "Animal feeding stuffs — Determination of vitamin A, E and D_3 content - method using solid phase extraction clean-up and High-Performance Liquid Chromatography" and to elaborate relevant method performance characteristics.

This report presents the design and the results of the method validation.

4 Study design

First step was preparation of the draft standard operating procedure. This document was distributed to members of WG3 group for commenting.

Following the trial was organized in two steps – pre-trail and main validation study.

4.1 Pre-trial

4.1.1 Design of pre-trial study

In pre-trail was distributed two samples from the Association of German Agricultural Analytic and Research Institutes (VDLUFA) proficiency testing. One sample was a complementary feed for milk cows and second was a mineral feed for piglets. Both feeds were produced by feed

industry. Complementary feed was granulated and was grounded in department of proficiency testing (PT) organisation ÚKZÚZ. The mineral feed was loose and was not grounded – Table A.

Table A: Nature of the tested materials and vitamin concentrations in the pre-trail

Test Material	**Sample Code**	**Declared concentration**		
		Vitamin A (IU/kg)	**Vitamin E** (mg/kg)	**Vitamin D** (IU/kg)
Complementary feed for milk cows	1 - KS	20 000	110	3 500
Mineral feed for piglets	2 - PX	300 000	2 500	50 000

Both samples were homogenised and tested for homogeneity of vitamin A and E. After homogenization, the feed materials were divided into sub-portions of 200 g and 70 g, respectively and stored by temperature 4 °C until distribution. The samples for the participants were randomly coded. Per material 12 randomly taken samples were used for homogeneity testing - Table B.

Table B: Summary of homogeneity tests (Anova F-test)

	Sample	Complementary feed for milk cows	Mineral feed for piglets
Vitamin A	F	0,469	3,419
Vitamin E	F	1,629	1,380
	F_{krit}	4,301	4,301

4.1.2 Sample distribution

Before pre-trial was started a Questionnaire about interest of participation in validation study was distributed in June 2017. 21 laboratories expressed their interest in participation in pre-trial (Annex 1 - List of participants pre-trial).

To laboratories that express interest was send “Call for participation in pre-trial” on 9.10.2017 and subsequently test samples.

Each of the participating laboratories received a randomly assigned laboratory code. The sample sets with the corresponding number, were sent to the participating laboratories on 10.10.2017. The sample sets were packed in a paper box at room temperature and were dispatched to the participants immediately by courier. The samples were accompanied by a letter describing the requested analyses and an acknowledgement of receipt form. By e-mail the laboratories received the method standard operating procedure, instructions on how to use the web application for reporting results and number of their laboratory.

Participants were invited to strictly follow the method and asked for three results per vitamin for each sample.

The main goal of the pre-trial was to give laboratories the opportunity to familiarize with the method and procure with the required equipment and supplies.

All comments and suggestions were used for the improvement of the method protocol prior to start the validation study.

4.1.3 Statistical evaluation

Results of the pre-trial were send by 16 laboratories.

Statistical evaluation of the results was carried out according to ISO 5725 and robust statistics according to DIN 38402-A45 (Annex 2) to compare both approaches.

Results for statistical evaluation of the pre-trail are shown in Annex 2.

4.2 Validation study

4.2.1 Design of validation study

The collaborative study was designed according to international guidelines, in particular to ensure minimum of 5 different matrix/analyte combinations for each analyte across the range of industrially produced feeds.

A full collaborative study was organised by ÚKZÚZ (Praha, Czech Republic) and the BfUL (Nossen, Germany) in January 2018.

Seven samples from VDLUFA and ÚKZÚZ proficiency testing were picked out. All feeds were produced by feed industry, samples of complete feed 1-KS and 3-KS were grounded and the other samples 2-PX, 4-PX, 5-KS, 6-KS and 7-PX were ungrounded. Besides these seven samples one sample of a standards mixture solution of vitamin A, D_3 and E was added. Nature of the tested materials and approximate vitamin concentrations are shown in Table C.

Table C: Nature of the tested materials and approx. vitamin concentrations

Test Material	Sample Code	Approx. concentration of parameters		
		Vitamin A (IU/kg)	Vitamin E (mg/kg)	Vitamin D (IU/kg)
Complete feed for broilers	1 - KS	10 000	30*	4 000
Premixture for broilers	2 - PX	4 000 000	10 000*	1 600 000
Complete feed for pigs	3 - KS	10 000	88*	2 000
Premixture for piglets	4 - PX	4 000 000	17 600*	800 000
Complete feed for turkey	5 - KS	10 000	100*	4 000
Complementary feed for cows	6 - KS	8 000	5*	1 500
Mineral feed	7 - PX	612 000	3 300*	132 000
Standard solution		19 – 21 IU/ml	11 – 13 µg/ml	D2: 70 – 80 IU/ml D3: 75 – 85 IU/ml

* Vitamin E in form of acetate

The materials 1 to 4 were prepared by the ÚKZÚZ (Praha, Czech Republic) and materials 5 to 7 by the BfUL (Nossen, Germany).

Samples were homogenised and tested for homogeneity of selected vitamins – Table D. After homogenization, the feed materials were divided into sub-portions and stored by temperature 4 °C until distribution. The samples for the participants were randomly coded. Per material 10 to 16 randomly taken samples were used for homogeneity testing.

Table D: Summary of homogeneity tests (Anova F-test)

	Sample	1 KS	2 PX	3 KS	4 PX	5 - KS	6 KS	7 PX
Vitamin A	F	0,003	3,419	2,736	1,368		6,144	0,169
Vitamin E	F	0,022	1,380	0,232	2,575	0,340	0,705	0,667
Vitamin D3	F	0,670	0,009			0,002	0,074	
	Fkrit	4,171	4,171	4,351	4,351	4,414	4,414	4,414

4.2.2 Sample distribution

Call for participation of the validation trial was distributed on January 2018, deadline for sending results end of February 2018 and evaluation of the results March 2018.

In total 17 laboratories took part in the validation study (Annex 3). The participating laboratories were familiarized with the method within a training study (pre-trail).

Each of the participating laboratories received a randomly assigned laboratory code. The sample sets with the corresponding number, were sent to the participating laboratories on 26th of January 2018. The sample sets were packed in a paper box at room temperature and were dispatched to the participants immediately by courier. The samples were accompanied by a letter describing the requested analyses and an acknowledgement of receipt form. By e-mail the laboratories received the draft of the standardized method (Annex 4), instructions on how to use the web application for reporting results and number of their laboratory (Annex 5) Participants were invited to strictly follow the method and asked for three results per vitamin for each sample. Because the vitamins are not stable a time frame for analysing the vitamins was defined. Laboratories were informed about the declared values of vitamin concentration or a range determined within the homogeneity test.

All 17 laboratories delivered results but not always for each vitamin or each methodical variation of saponification or detection.

4.2.3 Statistical evaluation of the results

The statistical evaluation was performed by the Saxon State Company for Environment and Agriculture (Nossen, Germany) in April 2018 using the statistics program PROlab of the company Quodata. The design of the study for the validation of the HPLC-method using SPE clean-up for determination the vitamins A, E and D_3 in feed samples was selected according to internationally accepted protocols for method validation.

First step of statistical evaluation was comparison of results from cold and hot saponification according to independent two-sample t-test. By this test was shown that there are no significant differences between both saponification procedures and therefore it is possible to evaluate the data together – Table E.

Graphical representation showing an Example of distribution of cold and hot saponification results is given in the Annex 6 “Example of graphical presentation of the results of the validation using the example of vitamin A in matrix 6-complementary feed for milk cows”.

Table E: Results of Independent two-sample t-test, Equal or unequal sample sizes

Sample code	1 - KS	2 - PX	3 - KS	4 - PX	5-KS	6-KS	7- PX
Vitamin A							
Observations Hot- / cold saponification	12/11	11/10	11/11	11/10	11/11	10/10	11/10
t_{stat}	**0,439**	**0,411**	**0,943**	**0,255**	**0,600**	**0,767**	**0,601**
$t_{krit\ (2)}$	**2,080**	**2,131**	**2,110**	**2,093**	**2,110**	**2,145**	**2,093**
Vitamin E							
Observations Hot- / cold saponification	12/12	11/11	11/12	11/11	11/12	10/11	11/11
t_{stat}	**1,163**	**0,846**	**0,162**	**1,097**	**0,530**	**0,109**	**0,545**
$t_{krit\ (2)}$	**2,074**	**2,086**	**2,093**	**2,093**	**2,179**	**2,160**	**2,131**
Vitamin D3							
Observations Hot- / cold saponification	6/9	6/9	5/9	6/9	5/9	4/8	6/9
t_{stat}	**0,364**	**0,687**	**0,014**	**0,159**	**0,399**	**0,844**	**0,168**
$t_{krit\ (2)}$	**2,262**	**2,228**	**2,306**	**2,228**	**2,571**	**2,776**	**2,179**

The quantitative results submitted by the laboratories were used to calculate performance characteristics (standard deviations under repeatability and reproducibility conditions) by applying ISO 5725 and the robust approach of DIN 38402-A45 – Table F.

Table F: Comparison two ways of statistic evaluation ISO 5725-2 (outlier testing) and DIN38402 A45 (robust statistic)

Vitamin A	**1-KS**	**2-PX**	**3–KS**	**4-PX**	**5-KS**	**6-KS**	**7-P X**
number of participants	24	22	23	22	23	21	22
Mean ISO (IU/kg)	9220	4140933	10808	3861802	9698	4461	565456
Mean DIN (IU/kg)	9209	4118352	10845	3861411	9677	4365	566315
SD_r ISO (IU/kg)	738	282680	645	201160	1355	706	35414
SD_r DIN (IU/kg)	569	206045	550	174653	1221	409	32798
RSD_r (repeatability) ISO (%)	8,00	6,83	5,97	5,21	13,97	15,83	6,26
RSD_r (repeatability) DIN (%)	6,18	5,00	5,07	4,52	12,62	9,37	5,79
SD_R ISO (IU/kg)	1115	493700	1079	358110	1858	1098	57201
SD_R DIN (IU/kg)	1140	465118	1171	365416	1999	770	61611
RSD_R (reproducibility) ISO (%)	12,09	11,92	9,98	9,27	19,16	24,61	10,12
RSD_R (reproducibility) DIN (%)	12,37	11,29	10,80	9,46	20,66	17,64	10,88

Vitamin E	**1-KS**	**2-PX**	**3-KS**	**4-PX**	**5-KS**	**6-KS**	**7-PX**
number of participants	24	22	23	22	23	21	22
Mean (mg/kg) ISO	31	4 209	98	13 926	121	23	3 572
Mean (mg/kg) DIN	32	4 171	98	13 800	120	22	3 530
SD_r (mg/kg) ISO	2	304	4	1 083	5	1	302
SD_r (mg/kg) DIN	1	193	4	664	3	1	147
RSD_r (repeatability) (%) ISO	6,45	7,22	4,08	7,78	4,13	4,35	8,45
RSD_r (repeatability) (%) DIN	3,13	4,63	4,08	4,81	2,50	4,55	4,16
SD_R (mg/kg) ISO	6	795	13	2 666	19	4	676
SD_R (mg/kg) DIN	4	696	14	2 559	18	4	579
RSD_R (reproducibility) (%) ISO	19,35	18,89	13,27	19,14	15,70	17,39	18,92
RSD_R (reproducibility) (%) DIN	12,50	16,69	14,29	18,54	15,00	18,18	16,40

Vitamin D3	**1-KS**	**2-PX**	**3-KS**	**4-PX**	**5-KS**	**6-KS**	**7-PX**
number of participants	15	15	14	15	14	12	15
Mean (IU/kg) ISO	3 777	1 653 716	2 004	824 063	3 962	1 662	105 947
Mean (IU/kg) DIN	3 772	1 638 150	2 038	826 983	3 962	1 668	104 680
SD_r (IU/kg) ISO	627	98 140	381	33 614	662	311	9 479
SD_r (IU/kg) DIN	200	74 249	242	36 324	411	274	6 524
RSD_r (repeatability) (%) ISO	16,60	5,93	19,01	4,08	16,71	18,71	8,95
RSD_r (repeatability) (%) DIN	5,30	4,53	11,87	4,39	10,37	16,43	6,23
SD_R (IU/kg) ISO	752	232 280	497	102 978	928	563	18 135
SD_R (IU/kg) DIN	753	211 463	484	113 457	1 020	572	14 435
RSD_R (reproducibility) (%) ISO	19,91	14,05	24,80	12,50	23,42	33,87	17,12
RSD_R (reproducibility) (%) DIN	19,96	12,91	23,75	13,72	25,74	34,29	13,79

For following evaluation, the robust statistics was used because the performance data were very strong comparable to that calculated by the approach according ISO 5725. The advantage of the robust approach is that this statistical method is relatively independent of the influence of possible outliers and further the distribution of data does not play such a significant role as for e.g. a parametric approach. The results of robust statistics are in Table G.

Table G: Results of the interlaboratory study - Robust statistics DIN 38402 A45

Vitamin A	1-KS	2-PX	3-KS	4-PX	5-KS	6-KS	7-PX
Number results	24	22	23	22	23	21	22
Mean (IU/kg)	9 209	4 118 352	10 845	3 861 411	9 677	4 365	566 315
Mean (µg/kg)	2 763	1 235 506	3 254	1 158 423	2 903	1 310	169 895
SD_r (IU/kg)	569	206 045	550	174 653	1 221	409	32 798
RSD_r (repeatability) (%)	6,18	5,00	5,07	4,52	12,62	9,37	5,79
SD_R (IU/kg)	1 140	465 118	1 171	365 416	1 999	770	61 611
RSD_R (reproducibility) (%)	12,37	11,29	10,80	9,46	20,66	17,64	10,88
RSD_R (reproducibility) (%), est. accor. Horwitz	13,73	5,48	13,40	5,53	13,63	15,36	7,40
HorRat	**0,90**	**2,06**	**0,81**	**1,71**	**1,52**	**1,15**	**1,47**

Vitamin E	1-KS	2-PX	3-KS	4-PX	5-KS	6-KS	7-PX
Number results	24	22	23	22	23	21	22
Mean (mg/kg)	32	4 171	98	13 800	120	22	3 530
SD_r (mg/kg)	1	193	4	664	3	1	147
RSD_r (repeatability) (%)	3,13	4,63	4,08	4,81	2,50	4,55	4,16
SD_R (mg/kg)	4	696	14	2 559	18	4	579
RSD_R (reproducibility) (%)	12,50	16,69	14,29	18,54	15,00	18,18	16,40
RSD_R (reproducibility) (%) estim. accor. Horwitz	9,50	4,56	8,02	3,81	7,78	10,05	4,68
HorRat	**1,32**	**3,66**	**1,78**	**4,87**	**1,93**	**1,81**	**3,50**

Evaluation of results for determination of vitamin E in premixtures and the mineral feed (2-PX, 4-PX and 7-PX) gives unsatisfactory HorRat values (table above). This led to thorough inspection of comments provided by laboratories. Non-statistical reasons for elimination of results of laboratories No. 2, 10, 12 and 20 for results of determination of vitamin E in premixtures and the mineral feed were found:

- laboratory no. 2 deviated from the protocol. They use always a 150 ml SPE-column. Use of such a column was only allowed for low concentrations of vit D3;
- laboratory no. 10 had problem with analysis the standard solution, so their results are questionable.
- laboratory no. 12 deviated from the protocol using 30ml columns instead of and 70ml columns for determination of vitamin E in samples 2-PX, 4-PX and 7-PX.
- laboratory no. 20 reported chromatographic problems with vit E **only** in sample 4-PX.

Recalculation after elimination of above-mentioned results gives HorRat values that became satisfactory and were accepted by WG3 members of CEN TC 327. Recalculated results for vitamin E were shown in the table below.

Vitamin E	1 - KS	2 - PX	3 - KS	4 - PX	5 - KS	6 - KS	7 - PX
Number results	21	18	20	17	20	18	18
Mean (mg/kg)	32,0	4250	99,0	14282	120	23,0	3581
SD_r (mg/kg)	1,0	193	3,0	808	3,0	1,0	137
RSD_r (repeatability) (%)	3,25	4,55	3,43	5,66	2,82	4,02	3,83
SD_R (mg/kg)	3,0	575	12,0	1723	17,0	3,0	462
RSD_R (reproducibility) (%)	10,49	13,54	12,09	12,06	14,28	14,59	12,90
RSD_R (reproducibility) (%) estim. accor. Horwitz	9,50	4,55	8,01	3,79	7,78	9,98	4,67
HorRat	**1,10**	**2,98**	**1,51**	**3,18**	**1,84**	**1,46**	**2,76**

Vitamin D_3	1-KS	2-PX	3-KS	4-PX	5-KS	6-KS	7-PX
Number results	15	15	14	15	14	12	15
Mean (IU/kg)	3 772	1 638 150	2 038	826 983	3 962	1 668	104 680
Mean (µg/kg)	94,0	40 954	51,0	20 675	99,0	42,0	2 617
SD_r (IU/kg)	200	74 249	242	36 324	411	274	6 524
RSD_r (repeatability) (%)	5,30	4,53	11,88	4,39	10,37	16,43	6,23
SD_R (IU/kg)	753	211 463	484	113 457	1 020	572	14 435
RSD_R (reproducibility) (%)	19,96	12,91	23,75	13,72	25,74	34,29	13,79
RSD_R (reproducibility) (%), est. accor. Horwitz/Thompson	22,00	9,15	22,00	10,14	22,00	22,00	13,84
HorRat (if mean<120µg/kg according THOMPSON)	**0,91**	**1,41**	**1,08**	**1,35**	**1,17**	**1,56**	**1,00**

The concentration of vitamins in standard solution was analysed in laboratories on the defined date - 5.2.2018. The results show the importance of calibration step and give an impression of the influence of this parameter on the results of the validation study. Improvement of this step could lead to lower reproducibility standard deviation, but this step was not planned to investigate during the validation study. The results of analysis standard solution are in Table H.

Table H: Results of measurement concentration of vitamin A, E and D_3 in the standard solution

	Mean	Min	Max	RSD (%)
Vit A (IU/ml)	19,7	17,8	24,5	8,23
Vit E (µg/ml)	12,4	10,1	17,5	14,92
Vit D_2 (IU/ml)	9,7	7,3	12,1	18,13
Vit D_3 (IU/ml)	9,8	7,8	13,2	16,39

Further graphic expression of dependence repeatability/ reproducibility to concentration was carry out and this dependence was calculated by equation:

Table I: Equation for dependence of repeatability and reproducibility on concentration

	Repeatability	**Reproducibility**
Vitamin A (IU/kg)	$y = 0,0479x$; $R^2 = 0,9956$	$y = 0,1044x$; $R^2 = 0,9888$
Vitamin E (mg/kg)	$y = 0,0547x$; $R^2 = 0,9889$	$y = 0,1222x$; $R^2 = 0,9983$
Vitamin D_3 (IU/kg)	$y = 0,0451x$ $R^2 = 0,9991$	$y = 0,1308x$; $R^2 = 0,9991$

Graphics showing the concentration dependence of repeatability/reproducibility are present in the Annex 6.

4.2.4 Results interpretation

The detailed results of individual laboratories and an example of graphical presentation of these data are shown in Annex 6.

Besides the results laboratory sent also identification of detection. Different detection technics have not significant influence on the results of vitamins A and E. One laboratory used MS/MS detection for determination of vitamin D_3 and even this technic gives comparable results. Because of only one result of MS/MS technic it is not possible to conclude that method is completely validated for this technic, but laboratories can verify this procedure in their laboratories.

Participating laboratories further shown analytical details connected to determination of vitamin A, E and D. These details are in Annex 7.

To show the fitness for purpose the HorRat values were calculated. HorRat values ≤ 2 can confirm the fitness of the method for the purpose.

The results for vitamin A and vitamin D_3 give acceptable HorRat value for all materials.

For vitamin E acceptable HorRat values were calculated for following materials: complete feed for broilers (material 1-KS), complete feed for pigs (material 3-KS), complete feed for turkey (material 5-KS) and complementary feed for milk cows (material 6-KS).

Some results for materials with high concentration of vitamin E - mineral premixtures for broilers (material 2-PX), mineral premixtures for piglets (material 4-PX) and mineral mixture for cows (material 7-PX) had to be removed for non-statistical reasons before data evaluation (see point 4.2.3).

Finally, for these materials with high vitamin E concentration - mineral premixtures for broilers (material 2-PX), mineral premixtures for piglets (material 4-PX) and mineral mixture for cows (material 7-PX) the HorRat values were slightly higher than 2 but RSD_R was lower than 15 %. Therefore, also for these materials is the method fully validated as WG3 of CEN TC 327 recommended the RSD_R as a further criterion in such cases. It was decided that the method is considered fit-for-the-purpose for a material if the RSD_R is <25%.

5 Summary and conclusion

The aim of this study was to conduct an interlaboratory trial for validation of the method "Determination of vitamin A, E and D content - method using solid phase extraction clean-up and High-Performance Liquid Chromatography".

Twenty-three laboratories from different countries of Europe applied for participation in this validation study, seventeen laboratories provided results at the end of validation trial.

The content of three vitamins in different concentration levels and in different matrices was analysed in triplicates.

For evaluation of the results were chosen robust statistics to enable using all date and simultaneously give lower weight to extreme data. Before evaluation results of determination vitamin E in samples with high concentration (2-PX, 4-PX and 7-PX) results of some laboratories were removed for non-statistical reasons (compare point 4.2.3).

For evaluation of fitness-for-purpose were combined HorRat criterion and RSD_R which were calculated for each analyte in each matrix.

The proposed analytical method can be considered as fully validated and can be applied for determination of vitamin A, E and D3 in feedingstuffs tested within the validation study.

ANNEX 1 - List of participants of pre-trial

Laboratory name	**City**	**Country**
EC-JRC-IRMM, EURL FA control, Directorate F - Health, Consumers and Reference Materials	Geel	Belgium
ÚKZÚZ, NRL	Praha 5	Czech Republik
ÚKZÚZ, NRL	Opava	Czech Republik
ÚKZÚZ, NRL OSARK	Lípa	Czech Republik
The Danish Veterinary and Food Administration	Lystrup	Denmark
Finnish Food Safety Authority Evira	Helsinki	Finland
Service Commun des Laboratoires	Rennes	France
Thüringer Landesanstalt für Landwirtschaft (TLL), Referat 240, Organische Analytik	Jena	Germany
Federal Institute for risk assessment (BfR)	Berlin	Germany
LUFA Nord-West, Institut für Futtermittel	Oldenburg	Germany
Research Centre Weihenstephan for Brewing and Food Quality	Freising	Germany
LAVES Feed Institute Stade	Stade	Germany
Landesbetrieb Hessisches Landeslabor (LHL)	Kassel	Germany
Landeslabor Berlin-Brandenburg	Potsdam	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Geschäftsbereich 6, FB62	Nossen	Germany
Chemisches und Veterinäruntersuchungsamt Rhein-Ruhr-Wupper	Krefeld	Germany
Rikilt Wageningen University & Research	Wageningen	Nederland
National Laboratory for Feedingstuffs	Lublin	Poland
Laboratorio Arbitral Agroalimentario. S. Gral de Control y de Laboratorios Alimentarios. MAPAMA.	Madrid	Spain
Laboratori Agroalimentari	Cabrils	Spain
Agroscope	Posieux	Switzerland

ANNEX 2 - Results of statistical evaluation for pre-trial

Vitamin A	**Complementary feed for milk cows**		**Mineral feed**	
	ISO	DIN	ISO	DIN
Number of participants	25	25	21	25
Mean (IU/kg)	13 935	13 932	255 218	259 366
SD_r (IU/kg)	2 313	1 458	29 353	29 733
RSD_r (repeatability) (%)	16,60	10,47	11,50	11,46
SD_R (IU/kg)	3 956	4 390	64 681	72 429
RSD_R (reproducibility) (%)	28,39	31,51	25,34	27,93

Vitamin E	**Complementary feed for milk cows**		**Mineral feed**	
	ISO	DIN	ISO	DIN
Number of participants	23	25	22	25
Mean (mg/kg)	95,5	94	2 198	2209
SD_r (mg/kg)	4,5	3,5	144	137
RSD_r (repeatability) (%)	4,71	3,72	6,55	6,20
SD_R (mg/kg)	27	24	567	556
RSD_R (reproducibility) (%)	28,27	25,53	25,80	25,17

Vitamin D_3	**Complementary feed for milk cows**		**Mineral feed**	
	ISO	DIN	ISO	DIN
Number of participants	13	16	12	15
Mean (IU/kg)	2750	2850	51 369	51 994
SD_r (IU/kg)	304	480	6 102	5 857
RSD_r (repeatability) (%)	11,05	16,84	11,88	11,26
SD_R (IU/kg)	1 028	1 762	12 488	13 980
RSD_R (reproducibility) (%)	37,38	61,82	24,31	26,89

ANNEX 3 – List of participants of validation study

Laboratory name	**City**	**Country**
EC-JRC-IRMM, EURL FA control, Directorate F - Health, Consumers and Reference Materials	Geel	Belgium
ÚKZÚZ, NRL	Praha 5	Czech Republic
ÚKZÚZ, NRL	Opava	Czech Republic
ÚKZÚZ, NRL OSARK	Lípa	Czech Republic
Finnish Food Safety Authority Evira	Helsinki	Finland
Service Commun des Laboratoires	Rennes	France
Thűringer Landesanstalt fűr Landwirtschaft (TLL), Referat 240, Organische Analytik	Jena	Germany
Federal Institute for risk assessment (BfR)	Berlin	Germany
LUFA Nord-West, Institut für Fittermittel	Oldenburg	Germany
Landesbetrieb Hessisches Landeslabor (LHL)	Kassel	Germany
Landeslabor Berlin-Brandenburg	Potsdam	Germany
National Laboratory for Feedingstuffs	Lublin	Poland
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Geschäftsbereich 6, FB62	Nossen	Germany
Chemisches und Veterinäruntersuchungsamt Rhein-Ruhr-Wupper	Krefeld	Germany
Rikilt Wageningen University & Research	Wageningen	Netherlands
Laboratori Agroalimentari	Cabrils	Spain
Agroscope	Posieux	Switzerland

ANNEX 4 – Standard operating procedure

CEN/TC 327

Secretariat: NEN

Animal feeding stuffs — Determination of vitamin A, E and D content -method using solid phase extraction clean-up and High-Performance Liquid Chromatography

Einführendes Element — Haupt-Element

Élément introductif — Élément central

ICS:

Descriptors:

Foreword

This document (EN XXXX:20XX) has been prepared by Technical Committee CEN/TC 327 “Animal feeding stuffs”, the secretariat of which is held by NEN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by xxx, and conflicting national standards shall be withdrawn at the latest by xxx.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association.

WARNING — The use of this protocol involves hazardous materials, operations and equipment. This protocol does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This International Standard specifies a method for the determination of the content of the total vitamin A (retinol), vitamin E (α-tocopherol) and vitamin D_3 (cholecalciferol) content in animal feed using solid phase extraction (SPE) clean-up and high-performance liquid chromatography (HPLC).

Note: The procedure enables also determination of vitamin D_2 but in this case another internal standard must be used. The method is fully validated only for vitamin D_3.

The method is validated for the following concentrations:

Vitamin A: 4365 – 4118352 IU/kg

Vitamin E: 22 – 13800 mg/kg

Vitamin D_3: 1668 – 1638150 IU/kg

The following limits of quantification should be achievable by UV-detection but must be validated by the user:

Vitamin A: 2000 IU/kg

Vitamin E: 10 mg/kg

Vitamin D_3: 1000 IU/kg

NOTE: Lower limits of quantification could be reachable using fluorescence detection (see observation 11.7).

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696:1987, Water for analytical laboratory use — Specification and test methods.

ISO 6498:2012, Animal feeding stuffs — Guideline for sample preparation.

3 Terms and definitions

For the purposes of this International Standard, the following terms and definitions apply.

3.1 Vitamin A (retinol) content

Content of all-trans-retinol and cis-isomers determined in accordance with this International Standard.

The vitamin A (retinol) content is expressed in International Units per kilogram (IU/kg).

1 IU of vitamin A (retinol) is equal to 0,300 µg of all-trans-retinol or 0,344 µg all-trans-retinol acetate or 0,546 µg all-trans-retinol palmitate or 0,359 µg all-trans-retinol propionate.

3.2 Vitamin E (α-tocopherol) content

Content of α-tocopherol determined in accordance with this International Standard. The content of vitamin E (α-tocopherol) could be also expressed as mg α-tocopheryl acetate per kg.

1 mg vitamin E (α-tocopherol) corresponds to 1,098 mg vitamin E acetate (α-tocopheryl acetate).

NOTE: In samples can also be present β-, γ-, δ-tocopherol and α-, β-, γ-, δ-tocotrienol. This method uses reverse phase separation which does not separate individual forms of tocopherol. Therefore, the content of vitamin E expressed as α tocopherol or α-tocopheryl acetate includes all forms without considering differences in vitamin activities and the respective proportions of each form. Using a normal phase-column the separation of α-, β-, γ- and δ-tocopherol is possible (see observation 11.6).

3.3 Vitamin D content

Vitamin D exists in form of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol). For feeding stuffs is only vitamin D_3 authorised as Feed Additives pursuant to Regulation (EC) No 1831/2003 and addition of vitamin D_2 is not allowed. Therefore, the vitamin D_2 can be used as internal standard. For accurate calculation of the results it is important that the sample does not contain any other vitamin D_2 then that added as internal standard.

3.3.1 Vitamin D_3 (cholecalciferol) content

The content of cholecalciferol determined in accordance with this European Standard. The content of vitamin D_3 is expressed in International Units per kg (IU/kg).

1 IU corresponds to an activity of 0,025 µg vitamin D_3 (cholecalciferol).

4 Principle

The sample is saponified with ethanolic potassium hydroxide solution. In case that vitamin D_3 (cholecalciferol) is to be determined the internal standard is added before saponification. The vitamins are extracted, purified by SPE column and eluted with cyclohexane. The cyclohexane is removed by evaporation and the residue is dissolved in methanol (for determination of vitamin A (retinol) and vitamin E (α-tocopherol)) or in n-hexane (for determination of vitamin D_3 (cholecalciferol)).

The vitamin A (retinol) and E (α-tocopherol) concentration in the methanolic extract is determined by reversed-phase liquid chromatography using external calibration and HPLC conditions that give a single peak for all retinol isomers as well as for all tocopherols.

The n-hexane extract for vitamin D_3 determination is purified by semi-preparative normal-phase HPLC on silica gel. The purified extract is separated by reversed-phase HPLC using conditions that give a baseline separation between the vitamin D_2 and vitamin D_3. Quantification of vitamin D_3 is performed by external standard calibration considering the recovery of the internal standard.

5 Reagents and materials

Use only reagents of recognized analytical grade.

5.1 Water, complying with at least grade 3 in accordance with ISO 3696:1991.

5.2 Potassium hydroxide, (KOH), w ≅ 85 %.

5.3 Ethanol (C_2H_5OH), w = 95 % (by volume), or equivalent industrial methylated spirit (ethanol denatured by methanol)

5.4 Ascorbic acid ($C_6H_8O_6$)

5.5 Ascorbic acid, solution, c = 200 g/l.

5.6 Sodium sulphide, ($Na_2S \times 9\ H_2O$)

5.7 Sodium sulphide, alkali solution (see 11.1 observations).

Dissolve 2000 g of potassium hydroxide (5.2) in 1200 ml of water (5.1). Dissolve 224 g of sodium sulphide (5.6) in 800 ml of water (5.1) in ultrasonic bath. Mix both solutions together.

Note: Dissolution of KOH is a slow procedure. It is necessary to mix the solution until as much as possible of KOH is dissolved. After addition of sodium sulphide solution, the residuum of KOH is dissolved.

5.8 2,6-Di-tert-butyl-4-methylphenol (BHT), (see 11.2 observations).

5.9 Inert gas, e.g. nitrogen.

5.10 Methanol, (CH_3OH), HPLC grade.

5.11 Ethanol, (CH_3CH_2OH), HPLC grade.

5.12 Cyclohexane, (C_6H_{12}).

5.13 2-Propanol, (C_3H_7OH).

5.14 n-Hexane, (C_6H_{14}).

5.15 Mobile phase for semi-preparative HPLC-clean up of vitamin D_3

Mixture of n-hexane (5.14) and propanol (5.13) in the proportions e.g. 98 + 2 (by volume). The ratio of the mixture must be adapted to the HPLC-column employed. If necessary, filter through a membrane filter (6.8).

5.16 Mobile phase for analytical HPLC

Mix together methanol (5.10) and water (5.1) in the proportions e.g. 98 + 2 (by volume). The exact ratio will be determined by the characteristics of the column employed. The use of other mobile phase composition is allowed provided the separation of vitamins according the scope of the method (1) is possible. If necessary, filter through a membrane filter (6.8).

5.17 Vitamin A standard substances.

5.17.1 **All-*trans*-retinol acetate,** ($C_{22}H_{32}O_2$), CAS = 127-47-9, MW = 328,49 g/mol, extra pure, of certified activity, e.g. 2,80 x 10^6 IU/g.

5.17.2 **All-*trans*- retinol palmitate,** ($C_{22}H_{32}O_2$), CAS = 79-81-2, MW = 524,86 g/mol, extra pure, of certified activity, e.g. 1,80 x 10^6 IU/g.

5.18 Vitamin E standard substance.

5.18.1 **DL-α-tocopherol,** ($C_{29}H_{50}O_2$), CAS = 10191-41-0, MW = 430,72 g/mol, extra pure, of certified activity.

5.19 Vitamin D standard substances.

5.19.1 **Vitamin D_2** (ergocalciferol; $C_{27}H_{44}O$), CAS = 50-14-6, MW = 384,62 g/mol, extra pure, of certified activity, e.g. 40 x10^6 IU/g.

5.19.2 **Vitamin D_3** (cholecalciferol; $C_{27}H_{44}O$), CAS = 67-97-0; MW = 384,62 g/mol, extra pure, of certified activity, e.g.40 x 10^6 IU/g.

5.20 Celite for SPE column

Base material coarse-grained kieselguhr (also known as diatomaceous earth, hydromatrix, celite); particle size: max. 10 % < 100 µm, max. 90 % < 500 µm, max. 5 % > 800 µm; large pore size, high pore volume, constantly high batch-to-batch quality.

6. Apparatus

Usual laboratory equipment and in particular, the following:

6.1 **Boiling water bath** with magnetic stirrer or electrical heating device with stirring (for hot saponification).

6.2 **Overturning rotating stirrer** (for cold saponification).

6.3 **Amber glassware** (see observation 11.3).

6.3.1 **Flat bottom - or conical flasks,** 250 and 500 ml, with ground-glass socket.

6.3.2 **Allihn condenser**, jacket length 300 mm, with ground-glass joint, with adapter for gas feed pipe.

6.3.3 **Graduated flasks with ground-glass stoppers,** narrow-necked, 20, 25, 50 and 100 ml.

6.3.4 **Pear shaped flask with ground-glass stoppers**, 100 ml.

6.4 **Vials**, suitable for sample concentrator.

6.5 **Column for SPE**,

filled with celite (e.g. Chromabond XTR, 70 ml volume) which is able to adsorb the water phase from the saponification solution (9.4.2) and release the vitamins A, E and D completely by elution

with organic solvents. The column shall have a capacity of not less than 20 ml aqueous solution and possibly closed by a valve at the outlet.

6.6 Rotary vacuum evaporator, with water bath at 40 °C.

6.7 Sample nitrogen concentrator, heated to 50 °C.

6.8 Membrane filter, compatible with methanol, 0,45 µm pore size e.g. Chromafil PET - 45/15 MS or suitable filter with smaller pore size.

6.9 HPLC system semi-preparative, for the clean-up of vitamin D, consisting of:

6.9.1 HPLC pump, set to deliver a constant eluent volume flow rate of e.g. 2,5 ml/min.

6.9.2 HPLC injection device, injection volume of 500 µl.

6.9.3 HPLC semi-preparative normal phase column with guard column (see 9.7.2).

6.9.4 Column oven, set to provide a constant column temperature.

6.9.5 UV-Detector

6.10 HPLC-system for analytical separation, consisting of the following:

6.10.1 HPLC-pump, set to deliver a constant eluent volume flow rate of e.g. 1 ml/min.

6.10.2 HPLC injection devices, injection volumes of 20 µl and 100 µl.

6.10.3 HPLC reversed-phase column, with guard column (see 9.8.1).

6.10.4 Column oven, set to provide a constant column temperature.

6.10.5 Detectors for UV- and fluorescent detection.

6.10.6 Integrator / data handling system.

6.11 UV (or UV-Visible) spectrophotometer, capable of measuring absorbance at the wavelengths defined in 9.2.1.3, 9.2.2.5, 9.2.2.6, 9.2.3.5 equipped with cells of 10 mm path length.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage. Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in EN ISO 6497:2002.

Store the sample in such a way that deterioration and change in its composition are prevented.

8 Sample preparation

Samples are grinded at the day of analysis as recommended in the Guidelines for sample preparation EN ISO 6498:2012.

Grind a portion of the well-mixed dry laboratory sample so that it passes through a sieve with 1 mm apertures. Prevent to heat up.

Do not grind the sample(s) if the particle size distribution is adequate (e.g. premixtures and concentrates).

Semi-moist pet foods (canned pet foods) homogenize by mincing.

Note: Samples can be ground before the day of analysis. In this case the storage conditions must prevent any degradation, e.g. freeze the ground sample and defrost it in a fridge a night before analysis.

9 Procedure

9.1 General

Because of the sensitivity of vitamins, A, E and D to UV radiation and air perform all operations away from natural and strong fluorescent light and as rapidly as is consistent with accurate working. Use amber glassware (6.3) where possible (see observation 11.3).

9.2 Preparation and standardization of standard solutions

9.2.1 Vitamin A (retinol)

For preparation of vitamin A (retinol) standard solutions use all-trans-retinol acetate (5.17.1) or all-trans-retinol palmitate (5.17.2).

Note: Standard substance of retinol itself is less stable then retinol palmitate or retinol acetate and therefor it is usual to use these esters for preparation of standard solution of vitamin A. Nevertheless, use of standard substance retinol is also possible.

9.2.1.1 Stock standard solution of vitamin A (retinol).

Weigh to the nearest 0,1 mg an amount of vitamin A (retinol acetate) (5.17.1) or vitamin A (retinol palmitate) (5.17.2) containing approximately 100 000 IU of vitamin A (retinol) into a 250 ml flat bottom or conical flask (6.3.1) and continue with saponification according to 9.4.2.1 or 9.4.2.2 (but without addition of BHT (5.8)) and extraction according to 9.5.

Collect the eluate from the SPE column (6.5) in a 100 ml graduated flask (6.3.3) and fill up to the mark with cyclohexane (5.12).

The nominal concentration of stock standard solution of vitamin A (retinol) in cyclohexane is approximately 75 IU per ml.

The exact content must be calculated from exact concentration of working standard solution of vitamin A (retinol) (9.2.1.2) determined according to 9.2.1.3.

The stock standard solution of vitamin A (retinol) is stable for 6 months in dark at 4°C and can be used for preparation of working standard solution according to 9.2.1.2 during this period.

9.2.1.2 Working standard solution of retinol.

Pipette 10,0 ml of the vitamin A (retinol) stock standard solution (9.2.1.1) into a 100 ml graduated flask and fill up to the mark with cyclohexane (5.12).

The nominal concentration of the working standard solution is 7,5 IU vitamin A (retinol) per ml.

The exact content of vitamin A (retinol) in working standard solution must be determined according to 9.2.1.3.

The working standard solution of vitamin A (retinol) is stable for at least 2 months in dark at 4°C but the real concentration must be checked before use.

9.2.1.3 Standardisation of the vitamin A (retinol) working standard solution (9.2.1.2)

The exact content of vitamin A (retinol) in working standard solution (9.2.1.2) is determined by spectrometric measurement. Measure the UV spectrum of this solution against cyclohexane (5.12) in the spectrophotometer (6.11) at the absorption maximum between 325 nm and 327 nm.

Calculation of the vitamin A (retinol) content:

Absorption coefficient of vitamin A (retinol) in cyclohexane $A_{1cm}^{1\%}$ = 1735, i.e. 1000 mg/100 ml

(= 33 333 IU/ml) provide absorption 1735 $\Rightarrow$ absorption of 7,5 IU/ml = 0,3904

$$C_A = \frac{33333 \times A_{wSTD}}{A_{325}} = \frac{33333 \times A_{wSDT}}{1735} = 19{,}212 \times A_{wSTD}$$

Where

C_A = IU vitamin A (retinol)/ml;

A_{325} = absorption coefficient of retinol in cyclohexane (1735);

A_{wSTD} = absorption of working standard solution (9.2.1.2);

33 333 = retinol concentration (in IU/ml) corresponds to 1% solution.

9.2.2 Vitamin E (α-tocopherol)

For preparation of vitamin E (α-tocopherol) standard solutions use DL-α-tocopherol (5.18.1).

Note: It is also possible to use as standard DL-α-tocopheryl acetate – see Annex B. Preparation from DL-α-tocopherol is preferable because in this case is not necessary to apply hydrolysis.

9.2.2.1 Stock standard solution of vitamin E (α-tocopherol)

Weigh 50 mg of vitamin E (DL-α-tocopherol) (5.18.1) to the nearest 0,1 mg, into a 100 ml graduated flask (6.2.3). Dissolve in ethanol (5.11) and make up to the mark with the same solvent.

The nominal concentration of this solution contains 500 μg vitamin E (α-tocopherol) per ml.

The exact content of vitamin E (α-tocopherol) in stock standard solution (9.2.2.1) has to be calculated from exact concentration of working standard solution of vitamin E (α-tocopherol) (9.2.2.2).

The stock standard solution of vitamin E (α-tocopherol) in ethanol is stable at least for six months in dark at 4 °C and can be used for preparation of working standard solution according to 9.2.2.2 during this period.

9.2.2.2 Working standard solution of vitamin E (α-tocopherol) in ethanol

Pipette 10,0 ml of the vitamin E (α-tocopherol) stock standard solution (9.2.2.1) into a 100 ml graduated flask and fill up to the mark with ethanol (5.11).

The nominal concentration of the working standard solution is 50 μg vitamin E (α-tocopherol) per ml.

The exact content of vitamin E (α-tocopherol) in working standard solution has to be determined according to 9.2.2.3.

The working standard solution of vitamin E (α-tocopherol) is stable for at least 2 months in dark at 4 °C but the real concentration must be checked before use.

9.2.2.3 Standardisation of the vitamin E (α-tocopherol) working standard solution in ethanol (9.2.2.2).

The exact content of vitamin E (α-tocopherol) in working standard solution (9.2.2.2) is determined by spectrometric measurement absorbance of working standard solution (9.2.2.2) in ethanol (5.11). Measure the UV spectrum of this solution against ethanol (5.11) in the spectrophotometer (6.11) at the absorption maximum between 250 nm and 320 nm. The absorption maximum shall be at 292 nm for ethanol (5.11).

Absorption coefficient of vitamin E (α-tocopherol) in ethanol (5.11) at 292 nm $A^{1\%}_{1cm}$ = 75,8 i.e. 1000 mg/100 ml provide absorption 75,8 ⇒ absorption of 50 μg/ml = 0,379.

Calculation of the vitamin E (α-tocopherol) content in ethanol (5.11)

$$C_E = \frac{10000 \times A_{wSTD}}{A_{292}} = 131{,}9 \times A_{wSTD}$$

Where

C_E = µg vitamin E (α-tocopherol)/ml

A_{292} = absorption coefficient of vitamin E (α-tocopherol) in ethanol (75,8)

A_{wSTD} = absorption of working standard solution (9.2.2.2)

10000 = vitamin E concentration (α-tocopherol) in µg/ml corresponds to 1% solution

9.2.3 Vitamin D

9.2.3.1 Stock internal standard solution of vitamin D_2 (ergocalciferol)

Weigh 50 mg vitamin D_2 (ergocalciferol) (5.19.1) to the nearest 0,1 mg into a 100 ml graduated flask (6.3.3), dissolve it in ethanol (5.14) and fill up to 100 ml with ethanol (5.11).

Nominal concentration of this solution is 20000 IU of vitamin D_2 (ergocalciferol) per ml.

The exact content of vitamin D_2 (ergocalciferol) in stock internal standard solution has to be determined according to 9.2.3.5.

The stock internal solution of vitamin D_2 (ergocalciferol) is stable for at least one month at 4 °C and can be used for preparation of internal working standard solution I according to 9.2.3.3 during this period.

9.2.3.2 Stock standard solution of vitamin D_3 (cholecalciferol)

Weigh 50 mg vitamin D_3 (cholecalciferol) (5.19.2) to the nearest 0,1 mg into a 100 ml graduated flask (6.3.3), dissolve it in ethanol (5.11) and fill up to 100 ml with ethanol (5.11).

Nominal concentration of this solution is 20000 IU of vitamin D_3 (cholecalciferol) per ml.

The exact content of vitamin D_3 (cholecalciferol) in standard solution must be determined according to 9.2.3.5.

The stock solution of vitamin D_3 (cholecalciferol) is stable for at least one month in dark at 4 °C and can be used for preparation of working standard solution I according to 9.2.3.3 during this period

9.2.3.3 Working standard solution I of the vitamin D_3 (cholecalciferol) and internal standard D_2 (ergocalciferol).

Transfer by pipette 5,0 ml of stock standard solutions of vitamin D_3 (cholecalciferol) (9.2.3.2) and 5,0 ml of stock internal standard solutions of vitamin D_2 (ergocalciferol) (9.2.3.1) in the same 100 ml graduated flask (6.3.3) and fill up to 100 ml with methanol (5.11).

The nominal concentration of working solution I of each vitamin D form (vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol)) is 1000 IU per ml.

This working standard solution must be freshly prepared before use.

9.2.3.4 Working standard solution II of the vitamin D_3 (cholecalciferol) and internal standard D_2 (ergocalciferol).

Pipette 1,0 ml of the working standard solution I (9.2.3.3) into 100 ml graduated flask (6.3.3), make up to the mark with methanol (5.13) and mix.

The nominal concentration of working solution II of each vitamin D form (vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol)) is 10 IU per ml.

The exact concentration has to be calculated from exact concentration of stock internal standard solution of vitamin D_2 (ergocalciferol) (9.2.3.1) or stock standard solution D_3 (cholecalciferol) (9.2.3.2).

This working standard solution II has to be freshly prepared before use.

9.2.3.5 Standardization of vitamins D_2 (ergocalciferol) internal stock standard solution and D_3 **(cholecalciferol) stock standard solution**

Dilute 2 ml of the vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) stock standard solutions (9.2.3.1 and 9.2.3.2) in separate graduated flasks (6.3.3) with ethanol (5.11) to 100 ml and measure the UV-spectrum from 220 nm to 320 nm against ethanol (5.11) on the spectrophotometer (6.11). The absorption maximum should be at 263 - 265 nm.

Absorption coefficient of vitamin D_2 (ergocalciferol) in ethanol (5.11) at 264 nm $A_{1cm}^{1\%}$ = 460; i.e. 1000 mg/100 ml (= 400000 IU/ml) provide absorption 460 ⇒ absorption of 400 IU/ml = 0,460.

Calculation of vitamin D_2 (ergocalciferol) content when measured in ethanol:

$$C_{D2} = \frac{400000 \times A_{STD}}{A_{264}} = \frac{400000 \times A_{SDT}}{460} = 869{,}6 \times A_{STD}$$

Where

C_{D2} = IU vitamin D_2 (ergocalciferol)/ml;

A_{264} = absorption coefficient of vitamin D_2 (ergocalciferol) in ethanol (460);

A_{STD} = absorption of stock standard solution (9.2.3.1) diluted according to 9.2.3.4.

Absorption coefficient of vitamin D_3 (cholecalciferol) in ethanol (5.11) at 264 nm $A_{1cm}^{1\%}$ = 485

i.e. 1000 mg/100 ml (=400000 IU/ml) provide absorption 485 ⇒ absorption of 400 IU/ml = 0,485.

Calculation of vitamin D_3 (cholecalciferol) content when measured in ethanol:

$$C_{D3} = \frac{400000 \times A_{STD}}{A_{264}} = \frac{400000 \times A_{SDT}}{485} = 824{,}7 \times A_{STD}$$

Where

C_{D3} = IU vitamin D_3 (cholecalciferol)/ml;

A_{264} = absorption coefficient of vitamin D_3 (cholecalciferol) in ethanol (485);

A_{STD} = absorption of stock standard solution (9.2.3.2) diluted according 9.2.3.5.

9.3 Calibration

9.3.1 General

Preferably carry out the preparation of the calibration solutions and the feedstuff samples at the same time. Nevertheless, the calibrations for vitamin A (retinol), vitamin E (α-tocopherol) and vitamin D_3

(cholecalciferol) can be used over a long time provided that the repeatability conditions in the laboratory are stable. This must be checked regularly by appropriate quality assurance procedures.

9.3.2 Calibration solutions and plotting of calibration graph for vitamins A (retinol) and E (α-tocopherol)

9.3.2.1 Evaporate a defined volume stock standard solution of vitamin A (retinol) (9.2.1.1) and working standard solution of vitamin A (retinol) (9.2.1.2) near to dryness under vacuum using a rotary vacuum evaporator (6.6) or sample concentrator (6.7) at a temperature not exceeding 40 °C and 50 °C respectively. When using vacuum evaporator, restore atmospheric pressure by admitting inert gas (5.9) finally. Dissolve the residue immediately in the same volume of methanol (5.10) taking for evaporation.

9.3.2.2 Transfer different volumes of the stock standard solution and working standard solution of vitamin A (retinol) (prepared according 9.3.2.1) and vitamin E (α-tocopherol) (9.2.2.1 or 9.2.2.2) into a series of 20 ml graduated flasks (6.3.3). Make up to the mark with methanol (5.11) and mix. Table 1 provides example for calibration solutions for determination of vitamins A and E.

The exact concentration of the calibration solutions should be calculated from concentration of working standard solutions of vitamin A (retinol) (9.2.1.2) and vitamin E (α-tocopherol) (9.2.2.2).

Table 1: Calibration solutions for vitamins A and E

Calibration solution	Volume of vitamin A (retinol) **working standard** solution in methanol (9.2.1.2) [ml]	Nominal concentration of vitamin A (retinol) [IU/ml]	Volume of vitamin E (α-tocopherol) **working standard** solution (9.2.2.2) [ml]	Nominal concentration of vitamin E (α-tocopherol) [µg/ml]
0	0,0	0,000	0,0	0,0
1	0,3	0,113	0,2	0,5
3	0,8	0,300	0,8	2,0
4	1,6	0,600	1,6	4,0
5	4,0	1,500	3,0	7,5
	Volume of vitamin A (retinol) **stock standard solution** in methanol (9.2.1.1) [ml]	Nominal concentration of vitamin A (retinol) [IU/ml]	Volume of vitamin E (α-tocopherol) **stock standard** solution (9.2.2.1) [ml]	Nominal concentration of vitamin E (α-tocopherol) [µg/ml]
6	0,8	3,00	0,6	15,0
7	1,6	6,00	1,2	30,0
8	4,8	18,00	2,4	60,0

NOTE: The calibration curve is linear for vitamin A (retinol) in the range 0,1 – 30 IU/ml and for vitamin E (α-tocopherol) 0,5 – 80 µg/ml but it is not necessary to prepare all concentrations in one calibration curve. It depends on the expected concentration of the test solution (9.6).

Inject 20 µl of each calibration solution several times and determine the mean peak heights (areas). Use a linear regression as mathematic model ($y = ax + b$), where x = exact content of vitamin in the calibration solutions and y = the corresponding mean peak height or area.

9.3.3 Calibration solution and plotting of calibration graph for vitamins D2 and D_3

Transfer different volumes of working standard solutions I (9.2.3.3) and working standard solutions II (9.2.3.4) into a series of 20 ml graduated flasks (6.3.3). Make up to the mark with methanol (5.11) and mix.

The exact concentration of the calibration solutions should be calculated from concentration of stock standard solutions of vitamin D_3 and stock internal standard solution of vitamin D_2 (9.2.3.1 and 9.2.3.2).

Table 2: Calibration solutions for vitamin D

	Volume of **working standard solution II** (9.2.3.4) [ml]	Nominal concentration of Vitamin D_2 or D_3 (IU/ml) in the calibration solution
1	0	0
2	2,00	1,00
3	6,00	3,00
4	12,0	6,00
	Volume of **working standard solution I** (9.2.3.3) [ml]	Nominal concentration of Vitamin D_2 or D_3 (IU/ml) in the calibration solution
5	0,2	10,0
6	0,4	20,0
7	1,0	50,0

Inject 100 µl of each calibration solution several times and determine the mean peak heights (areas).

Use a linear regression as mathematic model (y = ax + b), where x = exact content of vitamin in the calibration solutions and y = the corresponding mean peak height or area.

9.4 Analysis of the sample

9.4.1 Preparation of test sample

Mix the grinded sample (8) thoroughly and depending on the vitamin A (retinol) and/or vitamin E (α-tocopherol) and/or vitamin D_3 content weigh 2 g to 30 g to the nearest 1 mg of the sample (see observation 11.4) into a 500 ml flat bottom or conical flask (6.3.1).

Use vitamin D_2 as internal standard (ISTD).

Dilute the vitamin D_2 internal stock standard solution (9.2.3.1) so that 1 ml of the diluted sample solution contains the same amount of internal standard (IU) as the expected vitamin D_3 amount (IU) in the test portion. Pipette 1,0 ml of this diluted solution of vitamin D_2 into the flask with sample portion.

Use the added units of ISTD as $ISTD_{spiked}$ for recovery calculation according formula (4) (10.3). Close the saponification flask and wait for 10 minutes.

The addition of internal standard is unnecessary if only the vitamins A (retinol) and/or E (α-tocopherol) to be determined. In this case add 1 ml of ethanol (5.11) instead of internal standard solution. Nevertheless, the addition of internal standard does not affect the analysis of vitamin A (retinol) and/or vitamin E (α-tocopherol).

Proceed with 9.4.2.1 or 9.4.2.2.

NOTE: Annex A suggests examples for possible amounts of weighing combined with further aliquots and dilutions depending on the declared content in order to reach a concentration in the test solution which is within the calibration curve.

9.4.2 Saponification

9.4.2.1 Cold saponification

Add to the sample in conical or flat bottom flask approximately 1 g of ascorbic acid (5.4) and 100 mg BHT (5.8). Then add successively with swirling accurate volume of 130,0 ml ethanol (5.3) and finally exactly

30,0 ml alkali sodium sulphide solution (5.7) and mix well. Remove the air in the flask with a stream of nitrogen, close the flask and put it into overturning rotating stirrer (6.2) for at least 12 hours (preferably overnight) at the laboratory temperature.

9.4.2.2 Hot saponification

Add approximately 1 g of ascorbic acid (5.) and 100 mg BHT (5.8). Then add successively with swirling accurate volumes of 130,0 ml ethanol (5.3) and finally exactly 30,0 ml alkali sodium sulphide solution (5.7) and mix well. Fit a condenser (6.3.2) to the flask and immerse the flask in a water-bath with magnetic stirrer (6.1). Heat to boiling and allow refluxing for 30 minutes with stirring under a slow stream of inert gas (5.9). After that time separate the flask from the condenser and let the saponification solution cool to room temperature, e.g. in ice bath.

NOTE:In exceptional cases some products may require divergent saponification conditions (see observation 11.4) or a longer saponification time (see observation 11.5).

9.5 Extraction of vitamin A (retinol), vitamin E (α-tocopherol) and vitamin D_3 (cholecalciferol) by SPE

Be sure that the sample particles in the saponification solution (prepared according 9.4.2.1 or 9.4.2.2) were settling down and the supernatant is clear as far as possible. Transfer 40,0 ml of the supernatant into a 50 ml graduated flask (6.3.3). Fill up to the volume with ascorbic acid solution (5.5).
NOTE: The ascorbic acid solution (5.5) must be prepared freshly just before use.
Shake very thoroughly to forms a visually homogeneous emulsion and transfer 15,0 ml this solution immediately to each of two SPE columns (6.5) - one column for the determination of vitamin A (retinol) and/or vitamin E(α-tocopherol) and one for vitamin D_3 (cholecalciferol).
NOTE: The emulsion tends to rapidly disintegrate. Therefore, it is very important to transfer the aliquot of the well-shaken emulsion to the column immediately after the mixing.
Allow the solution to penetrate slowly into the column by gravity. After complete penetration of the extract into the column wait for 15 min. After this time add cyclohexane (5.12) on the column till the cyclohexane-front reach almost the outlet. Then wait for 5 min. Apply further volumes of cyclohexane (5.12) onto the column and collect the eluate that pass through the column by gravity into a 100 ml graduated flask (6.3.3). The flow rate should not be faster than 1 drop/sec. Stop eluting just before the defined volume (100 ml) is reached. Fill up to the volume with cyclohexane (5.12).

NOTE: For samples with low content of vitamin D_3 may be that the concentration in the eluate is too low for further determination.

The situation can be improved by transferring 15,0 ml of the solution on two 70 ml CHROMABOND columns (15 ml on each) and pooling of the eluates of the two columns.

9.6 Preparation of the test solution for HPLC separation

9.6.1 Vitamin A (retinol) and vitamin E (α-tocopherol)

Evaporate an aliquot of the cyclohexane extract (9.5) near to dryness under vacuum using a rotary vacuum evaporator (6.6) or sample concentrator (6.7) at a temperature not exceeding 40 °C and 50 °C respectively. This aliquot is taken into account for result calculation according the equations (1) and (2) as F_A. When using vacuum evaporator, restore atmospheric pressure by admitting inert gas (5.9) at the end.

For determination of vitamin A (retinol) and vitamin E (α-tocopherol) dissolve the residue immediately in a known volume (1 - 100 ml) of methanol (5.10). This volume is used as V_s for the result calculation according the equations (1) and (2) (10.1, 10.2).

NOTE: In the case that the residue remaining after evaporation is insoluble repeat the analysis with an increasing amount of alkali sodium sulphite solution (5.7) as described in observation 11.4.

The expected concentrations of vitamin A (retinol) and vitamin E (α-tocopherol) in the sample extract must be in the validated calibration range. If necessary, dilute the sample extract with methanol (5.10) to reach

this concentration range. This dilution is taken into account for result calculation according the equations (1) and (2) (10.1, 10.2) as F_D. Filter it through a membrane filter (6.8) and continue with HPLC determination according 9.8.

NOTE If delay is unavoidable, store the test solution under inert gas (5.9) in a refrigerator at a temperature of 4°C ± 1°C not longer than 24 hours. Before use allow it to return to room temperature in the dark.

9.6.2 Vitamin D_3 (cholecalciferol)

Evaporate an aliquot of the cyclohexane extract (9.5) near to dryness under vacuum using a rotary vacuum evaporator (6.6) or sample concentrator (6.7) at a temperature not exceeding 40 °C and 50 °C respectively. This aliquot is considered for result calculation according the equations (3) as F_A. When using vacuum evaporator, restore atmospheric pressure by admitting inert gas (5.9) at the end.

For determination of vitamin D_3 (cholecalciferol) remove the remaining solvent at the cyclohexane extract (9.5) with a stream of inert gas (5.9) and dissolve the residue immediately in 1 ml n-hexane (5.14).

Continue with semi-preparative clean-up according 9.7.

9.7 Semi-preparative HPLC clean-up for determination of vitamin D_3

9.7.1 Determination of retention time window for vitamin D_2/D_3 fraction

Pipette each 0,25 ml of the two vitamin D stock solutions (9.2.3.1 and 9.2.3.2) in a 100 ml pear shaped flask (6.3.4) and evaporate almost to dryness under reduced pressure on the vacuum rotary evaporator (6.6) at a temperature of max. 40 °C or by sample-nitrogen concentrator (6.7) at a temperature of max. 50 °C. Resolve the residue in 100 ml n-hexane (5.14). This standard solution has a concentration of 50 IU/ml of each vitamin D. Inject 500 µl of this standard several times on the column of the semi-preparative HPLC system (6.9) and determine the retention time window from the beginning to the end of the vitamin D peak. Calculate the average retention time windows and expand it by approximately 10% of the retention time of the peak start and the peak stop so that no loss of vitamin D may occur during the collection time.

9.7.2 Conditions for semi-preparative HPLC clean-up

Column: 250 mm x 8 mm, normal phase, e.g. MultoHigh 100 Si-5µ normal phase column, 250 x 8 mm ID with suitable guard column.

Mobile phase (5.18): mixture of n-hexane (5.14) and 2-propanol (5.13), e.g. in the proportions 98+2 (by volume). The mix ratio must be adjusted to the used column (6.9.3).

Flow rate: 2,5 ml/min

UV-detector: 265 nm

Injection volume: 500 µl

NOTE:Under normal phase conditions (9.7.2) vitamins D_2 and D_3 are not separated and both contained in the same semi-preparative fraction. Other conditions may be used provided that a satisfactory separation of vitamin D-fraction from other co-extractives is achieved. However, ensuring the constancy of those separation conditions with a significant impact on the retention time (column temperature, flow etc.) is particularly important to ensure that the collected fraction contains the vitamin D.

NOTE:Other techniques for the clean-up of the vitamin D are possible, e.g. thin layer chromatography (TLC) [4].

9.7.3 Collection of the vitamin D fraction

Apply 500 µl of the solution prepared according 9.6.2 on the column of the semi-preparative HPLC system (6.9) and elute with the mobile phase for preparative HPLC (5.15). Collect the fraction within the retention time window for vitamin D defined according (9.7.1) into a 50 ml pear shaped flask (6.3.4). Increase the flow rate by approximately 2 ml/min after separation of the vitamin D fraction and rinse the column for approximately 10 min to remove late eluting substances. Then adjust the original flow rate again.

Evaporate the collected solution with vitamin D fraction at reduced pressure on the vacuum rotary evaporator (6.6) or sample concentrator (6.7) at a temperature of max. 40 °C and 50 °C respectively, up to 2 - 3 ml and remove the rest of solvent in a stream of inert gas (5.9) to dryness. Resolve the residue immediately in 1,0 ml methanol (5.10) and if necessary use a short ultrasonic treatment. The expected concentration of vitamin D_3 must be in the range of calibration curve. If necessary, dilute the sample extract with methanol (5.10) to reach this concentration range. This dilution is taken into account for result calculation according the equations (3) and (4) as F_D. Filter it through a membrane filter (6.8) and use this solution for HPLC determination according 9.8.

NOTE: If delay is unavoidable, store the test solution under inert gas (5.9) in a refrigerator at a temperature of 4 °C ± 1 °C not longer than 24 hours. Before use allow it to return to room temperature in the dark.

9.8 High-performance liquid chromatography

9.8.1 Conditions for analytical HPLC

The following conditions are offered for guidance. Other conditions may be used provided that they give equivalent results, i.e. a satisfactory separation of vitamins A (retinol), D_2 (ergocalciferol), D_3 (cholecalciferol) and E (α-tocopherol) from each other and from other co-extractives is achieved.

Column: 250 mm x 4,6 mm, reversed-phase, C18, e.g. Hypurity C18, 150 x 4,6 mm, 5 µm (6.10.3) or Eclipse XDB-C8, 150 x 4,6 mm, 5 µm (see observation 11.6) with suitable guard column.

Mobile phase (5.19): Mixture of methanol (5.10) with water (5.1) in the proportions 980 + 20 (by volume). The mix ratio must be adjusted to the used column (6.10.3).

Flow rate: 0,5 ml/min.

Detector: UV-detector with variable wavelength adjustment allowing the measurement at wavelengths 325 nm for vitamin A (retinol), 292 nm for vitamin E (α-tocopherol) and 265 nm for vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) which is equipped with integrator/data handling system.

For determination of vitamin A (retinol), and E (α-tocopherol) alternatively a fluorescence detector (observation 11.7) can be used:

Vitamin A (retinol) - excitation wavelength 325 nm, emission wavelength 475 mm;

Vitamin E (α-tocopherol) - excitation wavelength 295 nm, emission wavelength 330 nm.

Injection volume: 20 µl (for vitamin A (retinol) and E (α-tocopherol); 100 µl (for vitamin D_3 (cholecalciferol)).

Retention times approx. 7 min for vitamin A (retinol), approx. 15 min for vitamin D_2 (ergocalciferol), approx. 16 min for vitamin D_3 (cholecalciferol) and approx. 18 min for vitamin E (α-tocopherol);

9.8.2 Determination

Inject requested volume (20 or 100 µl) of sample extract on the analytical column and measure the area (height) peaks of vitamins. Calculate the mean peak area (height) of several injections of the same sample extract. Determine the vitamins concentration by reference to calibration curves (9.3.2 and 9.3.3).

Use the calibration graph to calculate the vitamin A (retinol) and/or vitamin E (α-tocopherol) and/or vitamin D_2/D_3 (ergocalciferol/cholecalciferol) concentration from the heights (areas) determined in the sample extract by reference to calibration curves (9.3.2 and 9.3.3). The calculated values C were used in the equations (1), (2), (3) and (4) for final calculation of vitamins content in sample (10.1, 10.2 and 10.3).

10. Expression of results

10.1 Result calculation for vitamin A (retinol)

Calculate the numerical value of the vitamin A (retinol) content of the test sample by the equation:

$$(1) \qquad W_A = \frac{F_A \times C \times V_S \times F_D}{m} \times \frac{161}{40} \times \frac{50}{15} \times 1000 = \frac{F_A \times C \times V_S \times F_D \times 13471}{m}$$

where

W_A = numeric value of vitamin A (retinol) content of the test sample in IU/kg;

C = vitamin A (retinol) concentration of the test solution (9.8.2) in IU/ml;

V_s = volume of test solution (9.6.1), in ml;

F_A = 100 divided by the aliquot volume of the cyclohexane extract (9.5) taking for evaporation;

F_D = dilution factor of the test solution according to (9.6.1);

m = weight of the test portion in g;

The factor 13471 in formula (1) can be explained as follows.

C, Vs, F_A, F_D and m are variable values and therefore they are used as symbols in the formula.

The fixed values are:

161 = volume of the saponification solution (according 9.4.2.1 / 9.4.2.2) in ml;

40 = aliquot of the saponification solution (according 9.5) in ml;

50 = final volume of the aliquot of the saponification solution filled up with ascorbate solution (according 9.5) in ml;

15 = aliquot of this solution which is transferred to the SPE column (according 9.5) in ml;

1000 = conversion factor for weighing from g to kg.

Using the calculation way below the fixed values can be summarized to the factor 13417.

$$\frac{161}{40} \times \frac{50}{15} \times 1000 = 13471$$

Conversional factor from retinol to retinol acetate is 1,147.

Conversional factor from retinol to retinol-palmitate is 1,820.

Conversional factor from retinol to retinol-propionate is 1,195.

10.2 Result calculation for vitamin E (α-tocopherol)

a) Calculate the numerical value of the vitamin E (α-tocopherol) content of the test sample by the equation:

$$(2) \qquad W_E = \frac{F_A \times C \times V_s \times F_D}{m} \times \frac{161}{40} \times \frac{50}{15 \times 1000} \times 1000 = \frac{F_A \times C \times V_s \times F_D \times 13{,}417}{m}$$

where

W_E = numeric value of vitamin E (α-tocopherol) content of the test sample in mg/kg;

C = vitamin E (α-tocopherol) concentration of the test solution (9.8.2) in µg/ml;

F_A = 100 divided by the aliquot volume of the cyclohexane extract (9.5) taking for evaporation;

V_s = volume of test solution (9.6.1) in ml;

F_D = dilution factor of the test solution according to 9.6.1);

m = weight of the test portion in g;

The factor 13,417 in formula (2) can be explained as follows.

C, Vs, F_A, F_D and m are variable values and therefore they are used as symbols in the formula.

The fixed values are:

161 = volume of the saponification solution (according 9.4.2.1 / 9.4.2.2) in ml;

40 = aliquot of the saponification solution (according 9.5) in ml;

50 = final volume of the aliquot of the saponification solution filled up with ascorbate solution (according 9.5) in ml;

15 = aliquot of this solution which is transferred to the SPE column (according 9.5) in ml;

1000 = conversion factor for the result from µg (as calibrated) to mg;

1000 = conversion factor for weighing from g to kg.

Using the calculation way below the fixed values can be summarized to the factor 13,417.

$$\frac{161}{40} \times \frac{50}{15 \times 1000} \times 1000 = 13{,}471$$

Conversional factor from α-tocopherol to α- tocopheryl-acetate content is 1,1.

10.3 Result calculation for vitamin D_3 (cholecalciferol)

Calculate the numerical value of vitamin D_3 (cholecalciferol) content of the test sample by the equation:

$$(3)\quad W_D = \frac{F_A \times V_s \times C_{VitD} \times F_D}{m} \times \frac{161}{40} \times \frac{50}{15} \times \frac{1}{0{,}5} \times 1000 = \frac{F_A \times V_s \times C_{VitD} \times F_D \times 26833}{m}$$

where

W_D = numeric value of vitamin D_3(cholecalciferol) content of the test sample in international units per kg;

C_{VitD} = vitamin D_3(cholecalciferol) concentration of the test solution (9.8.2) in IU/ml;

F_A = 100 divided by the aliquot volume of the cyclohexane extract (9.5) taking for evaporation;

V_s = volume of test solution (9.7.3) = 1 ml;

F_D = dilution factor of the test solution according to 9.7.3;

m = weight of the test portion in g;

The factor 26833 in formula (3) can be explained as follows.

C_{VitD}, Vs, F_A, F_D and m are variable values and therefore they are used as symbols in the formula.

The fixed values are:

161 = volume of the saponification solution (according to 9.4.2.1 / 9.4.2.2) in ml;

40 = aliquot of the saponification solution (according to 9.5) in ml;

50 = final volume of the aliquot of the saponification solution filled up with ascorbate solution (according to 9.5) in ml;

15 = aliquot of this solution which is transferred to the SPE column (according to 9.5) in ml;

0,5 = injection volume into semi-prep. HPLC (9.7.3) in ml;

1 = resolve volume of the collected and evaporated vitamin D-fraction after semi-prep. HPLC (9.7.3) in ml;

1000 = conversion factor for weighing from g to kg.

Using the calculation way below the fixed values can be summarized to the factor 26 833.

$$\frac{161}{40}\times\frac{50}{15}\times\frac{1}{0{,}5}\times 1000 = 26833$$

Recovery of vitamin D

Calculate recovery based on the units of internal standard added to the test solution according to 9.4.1 by the equation (4):

$$(4) \qquad R = \frac{C_{ISTD}\times F_D\times F_A\times V_S}{ISTD_{spiked}} \times \frac{161}{40}\times\frac{50}{15}\times\frac{1}{0{,}5}\times 100 = \frac{C_{ISTD}\times F_D\times F_A\times V_S\times 2683{,}3}{ISTD_{spiked}}$$

Where

R = Recovery rate in percent;

C_{ISTD} = internal standard vitamin D_2 concentration of the test solution (9.8.2) in IU/ml;

F_A = 100 divided by the aliquot volume of the cyclohexane extract (9.5) taking for evaporation;

V_S = volume of test solution (9.7.3) = 1 ml;

F_D = dilution factor of the test solution according to 9.7.3;

$ISTD_{spiked}$ = spiked activity of internal standard vitamin D_2 (according to 9.4.1) in IU;

The factor in formula (4) can be explained as follows.

C_{ISTD}, Vs, F_A, F_D and m are variable values and therefore it will use as symbols in the formula.

The fixed values are:

161 = volume of the saponification solution (according to 9.4.2.1 / 9.4.2.2) in ml

40 = aliquot of the saponification solution (according to 9.5) in ml

50 = final volume of the aliquot of the saponification solution filled up with ascorbate solution (according to 9.5) in ml

15 = aliquot of this solution which is transferred to the SPE column (according to 9.5) in ml

0,5 = injection volume into semi-prep. HPLC (9.7.3) in ml

1 = resolve volume of the collected and evaporated vitamin D-fraction after semi-prep. HPLC (9.7.3) in ml

100 = conversion factor for recovery to express it in %

Using the calculation way below the fixed values can be summarized to the factor 2683,3.

$$\frac{161}{40}\times\frac{50}{15}\times\frac{1}{0,5}\times 100 = 2683,3$$

Because it is assumed that vitamin D_2 and vitamin D_3 behave in the same way during the analysis the recovery of vitamin D_2 can be used for correction of content of vitamin D_3.

Calculate the corrected value of vitamin D_3 (cholecalciferol) content in the test sample (calculated by equation 3) by the recovery rate of the internal standard vitamin D2 (calculated by equation 4) according equation (5):

$$(5) \qquad W_{Dcorr} = \frac{(W_D \times 100)}{R}$$

where

$W_{D\ corr.}$ = numeric value of vitamin D3 (cholecalciferol) content in the test sample corrected by the recovery rate of ISTD calculated by equation (5) in international units per kg;

W_D = numeric value of vitamin D3 (cholecalciferol) content of the test sample in International units per kg calculated by equation (3);

R = Recovery rate in percent calculated by equation (4).

NOTE: In the case that the chromatogram of the separation of vitamin D2 and D3 shows abnormalities (e.g. interference substances near the peak of vitamin D2) that raise doubts about the plausibility of the recovery rate calculated according equation (4) or in case of analysis of unknown matrices, prepare the test solution in double (one with and one without addition of ISTD) to be sure that none interference influence the recovery rate of vitamin D_2 ISTD.

11. Observations

11.1 Approximately 50 mg EDTA can be used instead of sodium sulphide (5.6).

11.2 Hydroquinone can be used instead of BHT (5.8).

11.3 If amber glass is not available vitamins could be protected against UV light by covering glassware with aluminium foil or working in an environment of Sodium lamp light or light filtered for the same conditions.

11.4 In cases of analysis of vitamin A (retinol) in high fatty matrices (e.g. milk replacers) specific attention has to be paid at saponification (9.4.2). Due to the amount of fat present in the sample increasing of alkali sodium sulphide solution (5.7) may be necessary at saponification. The additionally added amount of alkali sodium sulphide solution (5.7) has to be taken into consideration during result calculation (10) by changing the factors used in the equations.

11.5 With cod-liver oil and other pure fats the saponification time shall be extended to 45-60 minutes.

11.6 Using a normal phase column for analytical HPLC (9.8) the separation of tocopherol isomers (α-, β-, γ- , δ-tocopherol and α -, β-, γ- , δ-tocotrienols) is possible. This can be important if antioxidants with high content of γ-tocopherols are present in feed (e.g. pet food).

11.7 Fluorescent detection is more sensitive. Applying of two different detections may give analyst more information in case of some doubts.

13. Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this European Standard;

d) all operating details not specified in this European Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result obtained.

Bibliography

[1] EN ISO 14565 Animal feeding stuffs — Determination of vitamin A content —Method using high-performance liquid chromatography

[2] EN ISO 6867 Animal feeding stuffs — Determination of vitamin E content —Method using high-performance liquid chromatography

[3] Commission Regulation (EC) No 152/2009 of 27 January 2009, laying down the methods of sampling and analysis for the official control of feed, Annex IV/A and ANNEX IV/B

[4] VDLUFA – Methodenbuch III – 13.8.1 Bestimmung von Vitamin D_3 HPLC-verfahren

[5] EN 12821 — Foodstuffs – Determination of vitamin D by high performance liquid chromatography: Measurement of cholecalciferol (D3) or ergocalciferol (D2)

[6] EN 12822 — Foodstuffs – Determination of vitamin E by high performance liquid chromatography: Measurement of α-, β-, γ- and δ- Tocopherol

[7] EN 12823-1 — Foodstuffs – Determination of vitamin A by high performance liquid chromatography – Part 1: Measurement of all-trans-retinol and 13-cis retinol

[8] ISO 5725-1 — Accuracy (trueness and precision of measurement methods and results – Part 1: General principles and definitions

[9] ISO 5725-2 — Accuracy (trueness and precision of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

[10] ISO 5725-3 — Accuracy (trueness and precision of measurement methods and results – Part 3: Intermediate measures of the precision of a standard measurement method

[11] ISO 5725-4 — Accuracy (trueness and precision of measurement methods and results – Part 4: Basic methods for the determination of the trueness of a standard measurement method

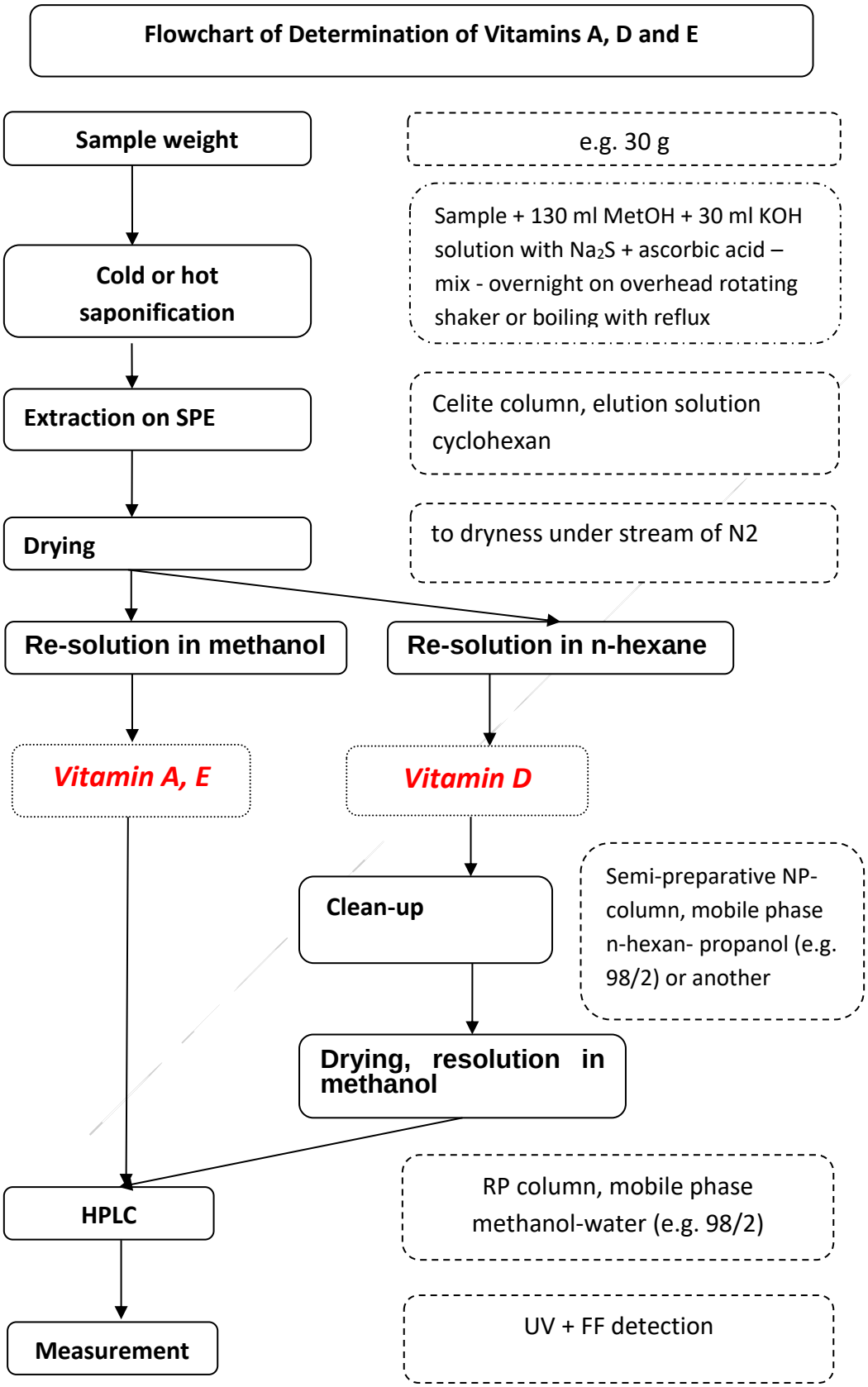
[12] ISO Animal feeding stuffs — Sampling.

Values of absorption coefficients A1%/1 cm) for vitamin E specified for a particular solvent, are published in literature (e.g. Mukai et. al.; Product sheet Sigma-Aldrich; Thurnham et al; ISO 6867, point

8.5.2; Tee et al; Craft Technologies Lab manual procedure; EU-regulation 152/2009, annex IV; Craft Technologies, Lab manual procedure; Bell et al; German official collection of methods for food analysis, method L00.00-62; Pharmacopeia; Merck Index …

DIN 38402 A45:2014 German standard methods for the examination of water, waste water and sludge - General information (group A) - Part 45: Interlaboratory comparisons for proficiency testing of laboratories (A 45)

Annex (informative)



Annex (informative)

Preparation of stock standard solution of vitamin E (α-tocopherol) from α-tocopheryl acetate ($C_{31}H_{52}O_3$, CAS 7695-91-2, MW = 472,76 g/mol, extra pure, of certified activity).

In difference to vitamin A (retinol) the alcohol form of vitamin E (α-tocopherol) is more stable. Therefore, it is used more often for preparation of the standard solution vitamin E (α-tocopherol), but it is also possible to use as standard substance α-tocopheryl acetate.

Preparation of Stock standard

Weigh approximately 73,3 mg of α-tocopheryl acetate to the nearest 0,1 mg into a 250 ml flat bottom or conical flask (6.3.1) and continue with saponification according 9.4.2.1 or 9.4.2.2 and extraction according 9.5.

Collect the eluate from the SPE column in a 100 ml graduated flask and fill up to the mark with cyclohexane (5.12).

The stock standard solution of vitamin E (α-tocopherol) is stable for 6 months in dark at 4° C and can be used for preparation of working standard solution according 9.2.1.2.

The nominal concentration of this stock standard solution contains 50,0 µg vitamin E (α-tocopherol) per ml.

The exact content has to be calculated according 9.2.2.2.

The stock solution of vitamin E (α-tocopherol) in cyclohexane is stable at least for six months in dark at 4 °C.

Standardisation of the vitamin E (α-tocopherol) stock standard solution in cyclohexane

The exact content of vitamin E (α-tocopherol) in the stock standard solution (9.2.2.1) is determined by spectrometric measurement. Measure the UV spectrum of this solution against cyclohexane (5.12) in the spectrophotometer (6.11) at the absorption maximum between 250 nm and 320 nm. The absorption maximum shall be at 292 nm for cyclohexane (5.12).

Absorption coefficient of vitamin E (α-tocopherol) in cyclohexane (5.12) at 292 nm $A_{1cm}^{1\%}$ = 84; i.e. 1000 mg/100 ml provide absorption 84 $\Rightarrow$ absorption of 50,0 µg/ml = 0,4202.

Calculation of the vitamin E (α-tocopherol) content when measured in cyclohexane:

$$C_E = \frac{10000 \times A_{wSDT}}{A_{292}} = 119{,}1 \times A_{wSTD}$$

Where

C_E = µg vitamin E (α-tocopherol)/ml;

A_{292} = absorption coefficient of vitamin E (α-tocopherol) in cyclohexane (84);

A_{wSTD} = absorption of stock standard solution (9.2.2.1);

10000 = concentration vitamin E (α-tocopherol) correspond to 1% solution in µg/ml.

ANNEX 5 - Instructions for validation.

1) Invitation letter

Central Institute for Supervising and Testing in Agriculture
ISO 9001:2008 certified

To all participants
of the CEN project M523/P2
validation study 2018 of vitamins

Date: 26. 01. 2018
Handled by: Jaroslava Petrová
Phone: +402 737 267 781
Mail: jaroslava.petrova@ukzuz.cz
ADRESS: Za Opravnou 4, 150 06 Praha 5 – Motol, Czech Republic

Validation study of the methods

„Determination of vitamin A, E and D content - method using solid phase extraction clean-up and High-Performance Liquid Chromatography"

Dear participant,

Thank you for your willingness to participate in the standardization process of the method "Determination of vitamin A, E and D content - method using solid phase extraction clean-up and High-Performance Liquid Chromatography".

This validation step is organized by the Central Institute for Supervising and Testing in Agriculture Prague and BfUL/LUFA Nossen within the 3rd Mandate of the European Commission to the European Standardization body CEN.

Design of the standardization process

In this validation study we will analyse seven samples and one standard solution. The main goal of this study is to validate suggested method and convert it into EN standard.

For validation of analytical method is very important **to strictly follow the procedure**. The valid method version is sending by @-mail together with this instruction.

Please special care takes for SPE column – use whenever possible pre-packed columns Chromabond XTR, 70 ml volume.

Deviations or problems during the analysis should be precisely documented and announced to organizers.

2.) Instruction sheet

1) Samples

a) Please check the content of the parcel carefully.

b) Please send the confirmation of receipt to e-mail: jaroslava.petrova@ukzuz.cz.

c) Sample of standard solution analyse **first on 5.2.2018. Shake it well before analysis. Results of this analysis write into the corresponding excel-sheet of the file *Analytical detail code.xlsx* -- "Results of standard solution".**

d) The samples of complete feed 1 KS and 3 KS are grounded and homogenized. Before analysis **mix every sample thoroughly** due to possible segregation during shipping.

d) The samples of complete feed 5 KS and 6 KS are homogenized but **not grinded**. Grind the whole package of the sample before analysis and **mix thoroughly** before weighing.

e) The samples of mineral feed and premixtures are ungrounded; ground the whole content just before analysis and **mix thoroughly** before weighing.

d) **Perform the analyses exactly as described in the method "3First Draft EN Vitamins with comments implemented_05_1_2018.docx"**. For analysis weight **30 g of complete feeds** (1-KS, 3 KS, 5 KS, 6 KS) and **10 g of mineral feeds** (2-PX, 4 PX, 7 PX).

e) Analyse **each sample in 3 replicates** (3 independent determinations in one day) following the **cold saponification** and **3 replicates** (3 independent determinations in one day) following the **hot saponification.** If you are not able to do one of the saponification carry on only one – but results from both are appreciated.

2) Reporting of results

a) For reporting your data, you will receive four report files – two for cold saponification and two for hot saponification. These files and "Annex for reporting" will send by e-mail in the next days. For each sample **3 replicates should be reported** in IU/kg and mg/kg (as specified in the report file).

Follow the instructions in "ANNEX FOR REPORTING".

b) You will also receive the same file "Analytical details" as in pre-trial. Please update information in this file. Comments, deviations or problems during the analysis should be also precisely documented in the "Analytical details" file.

Identify each single document with your laboratory name.

c) Lab code will be submitted electronically with report sheets.

Please return the report file with all data not later than February 28, 2018 to the following e-mail address: jaroslava.petrova@ukzuz.cz

The test samples sent by TNT service.

Contents of the package

Please find enclosed the study material consisting of:

- 3 samples of complete feeding stuff (appx. 250g);
- 1 sample of complementary feed (appx. 250g);
- 1 sample of mineral feed (appx. 100g);
- 2 samples of premixtures (appx. 100g);

The approximate content of vitamins is as follows:

Test Material	**Sample Code**	**Approx. concentration of parameters**		
		Vitamin A (IU/kg)	**Vitamin E** (mg/kg)	**Vitamin D** (IU/kg)
Complete feed for broilers	**1 KS**	**10 000**	**30 (acetate)**	**4 000**
Premixture for broilers	**2 PX**	**4 000 000**	**10 000 (acetate)**	**1 600 000**
Complete feed for pigs	**3 KS**	**10 000**	**88 (acetate)**	**2 000**
Premixture for piglets	**4 PX**	**4 000 010**	**17 600 (acetate)**	**800 000**
Complete feed for turkey	**5 KS**	**10 000 – 13 000**	**100 - 140**	**3 000 – 5 000**
Complementary feed for cows	**6 -KS**	**7 000 - 9 000**	**15 - 25**	**1 500 – 2 000**
Mineral feed	**7 - PX**	**550 000 – 700 000**	**4 000 – 5 000**	**150 000 – 200 000**
Standard solution		**19 – 21 IU/ml**	**11 – 13 μg/ml**	**D2 70 – 80 IU/ml D3 75 – 85 IU/ml/**

The SOP will be sent by email.

If you have any question regarding the method or validation process, please do not hesitate to contact by e-mail – jaroslava.petrova@ukzuz.cz

The deadline for reporting the results of the validation study is February 28, 2018.

We wish you a successful analysis of the samples!

Yours sincerely,

Jarka Petrová and Jens Schönherr

3.) Confirmation of receipt

Please find below the list of samples for the validation study for the standardization process „Determination of vitamin A, E and D content -method using solid phase extraction clean-up and High-Performance Liquid Chromatography"

Please check the content of the package carefully and return the completed form to

jaroslava.petrova@ukzuz.cz

Name: _____
Laboratory: _____
Address: _____
_____

Date of arrival of samples: _____
Date of checking samples: _____

Test Material	**Sample Code**	**Condition at Arrival**	
Complete feed for broilers	**1 KS**	□ o.k.	□ damaged
Premixture for broilers	**2 PX**	□ o.k.	□ damaged
Complete feed for pigs	**3 KS**	□ o.k.	□ damaged
Premixture for piglets	**4 PX**	□ o.k.	□ damaged
Complete feed for turkey	**5 KS**	□ o.k.	□ damaged
Complementary feed for cows	**6 -KS**	□ o.k.	□ damaged
Mineral feed	**7 - PX**	□ o.k.	□ damaged
Standard solution		□ o.k.	□ damaged

Comments:

Date: _____ Signature: _____

4.) Analytical details

Name of laboratory:

Sheet 1: Instructions

Lab code:

Dear Participants,

please, fill answers to following questions and send this file together with chromatograms of calibration solutions and samples as addition to result files. This excel file contains nine worksheets and should be sent back when filled in. Change the name of the file to Analytical details_xxx, where xxx is your code number. Lab code you will receive by e-mail with result sheets. Please fill in all coloured fields. Please use the "comments" text box for any additional information you wish to provide and/or to specify any issue. Please provide requested chromatograms with specified retention times and concentrations Please insert the asked chromatograms in this document or send them directly with this excel sheet.

The information collected will kept confidentially and only used for discussion and exchange during the standardisation process.

Sheet 2: **Saponification**

Name of laboratory:

Lab code:

Equipment for saponification	Type	Trade mark	Supplier
"Cold"			
"Hot"			

Comments:

Sheet 3: **Extraction**

Name of laboratory:

Lab code:

SPE used	Type	Size (ml)	Diameter (mm)	Trade mark	Supplier	Total volume of extraction solvent (ml)

Comments:

Sheet 4: **Semi-preparative column**

Name of laboratory:

Lab code:

Semi-preparative column for vitamin D	Phase	Length (mm)	Diameter (mm)	Particles (um)	Trade mark	Supplier

Comments:

Sheet 5: **Standards**

Name of laboratory:

Lab code:

Standards	Form (alcohol, ester)	CAS No.	Supplier
Standard vitamin A			
Standard vitamin E			
Standard vitamin D			

Comments:

Sheet 6: **HPLC**

Name of laboratory:
Lab code:

	Type	Supplier
HPLC		
UV detector		
FL detector		
Analytical column	Type	Phase
injection volume		µl
flow rate		ml min^{-1}
column temperature		°C

Comments:

Sheet 7: **Calibration Standards**

Name of laboratory:
Lab code:

Standard stock solution:

	purity	supplier	weighed mass (mg)	concentration (µg/ml)
Vitamin A				
Vitamin E				
Vitamin D				

Calibration standards:

Vit A	Concentration	Area	Vit E	Concentration	Area	Vit D	Concentration	Area

calibration curve (e.g. y=bx+a):
r-square:

calibration curve (e.g. y=bx+a):
r-square:

Comments:

Sheet 8: **Chromatogram**

Name of laboratory:
Lab code:

Please insert following chromatograms:
1. Calibration solutions
2. Samples
You can insert copy of chromatograms on this sheet or send it separately.

Sheet 9: **Recovery**

Name of laboratory:
Lab code:

Recovery for calculation of corrected value of vitamin D_3

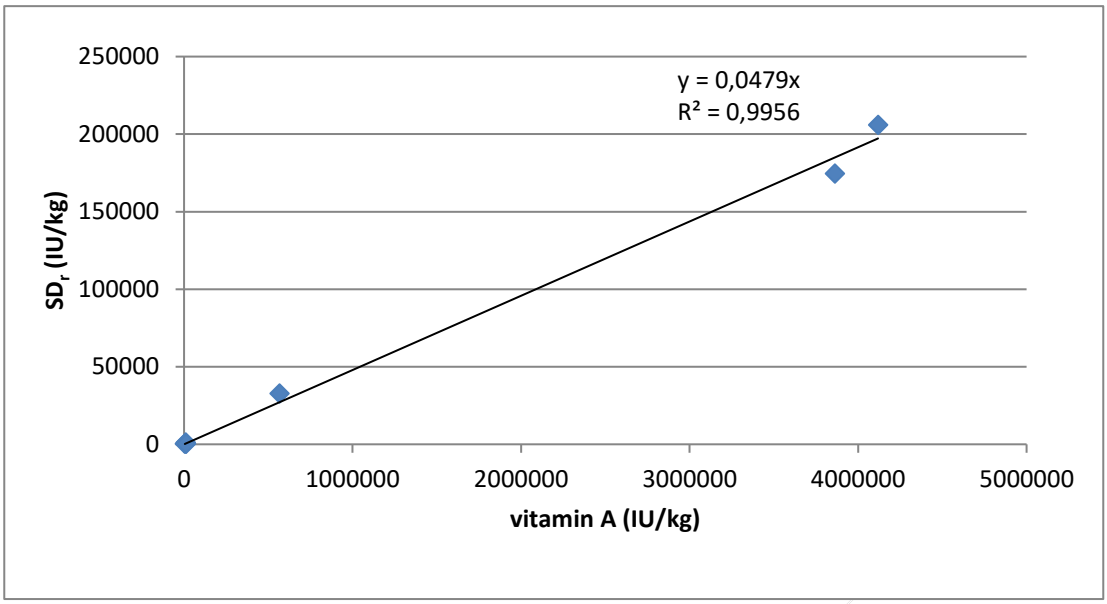
	Value 1	Value 2	Value 3
Sample 2-PX			
Sample 1-KS			

ANNEX 6– Results of the interlaboratory study

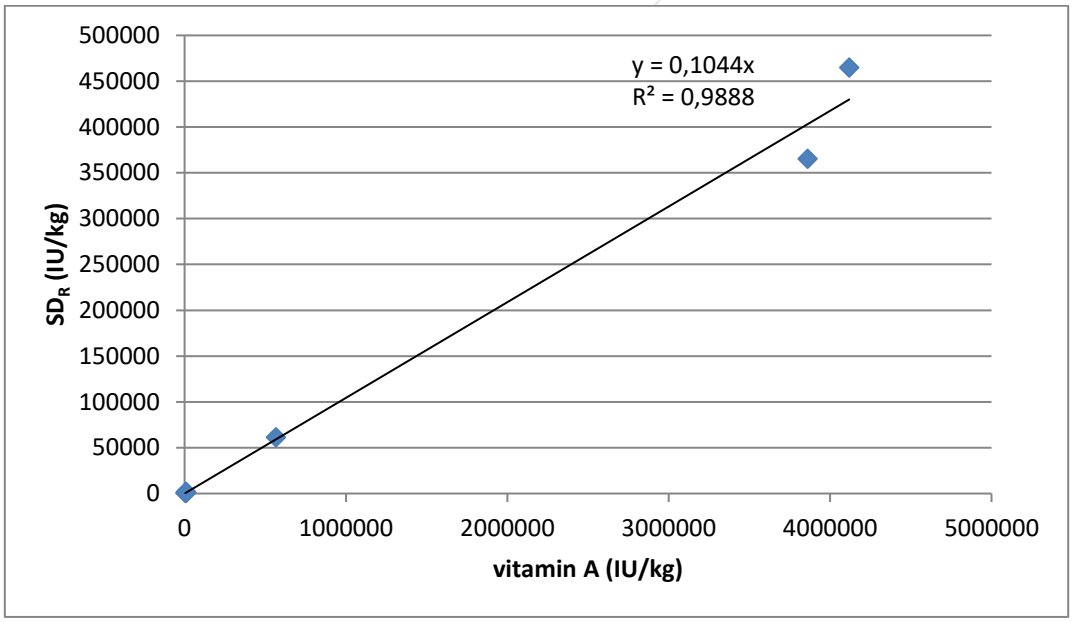
Vitamin A

	1 - KS	2 - PX	3 - KS	4 - PX	5- KS	6- KS	7 - PX	Detection method
Unit	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	
01-HOT	8 151	3 709 307	9 714	4 385 910	10 327	3 836	486 605	UV
02-COLD	8 750	4 021 571	10 792	3 989 184	10 211	4 084	554 066	UV
02-HOT	9 526	4 692 834	11 018	4 243 884	8 584	4 638	583 311	UV
04-COLD	8 373	3 953 333	11 000	3 716 667	8 627	4 773	603 333	UV
04-HOT	9 680	4 223 333	11 567	3 856 667	7 437	4 367	633 333	UV
07-COLD	10 185							UV
07-HOT	9 913	4 322 067	11 151	3 573 333	12 277	5 151	562 533	UV
09-HOT	9 287	4 090 000	11 200	3 666 667	13 167	4 123	550 667	UV
10-COLD	8 904	3 918 605	10 443	3 701 081	9 446	4 074	602 071	UV
12-COLD	10 011	4 345 927	12 570	4 015 647	10 432	5 118	549 252	UV
12-HOT	9 572		10 301		9 243	4 410		UV
13-COLD	8 620	4 076 505	10 622	4 418 149	10 309		604 875	UV
13-HOT	9 786	4 038 451	11 244	4 011 517	9 554		638 032	UV
14-COLD	8 429	4 082 291	9 779	3 481 190	9 131	3 647	455 007	UV
15-COLD	11 130	3 772 463	12 170	3 982 592	10 411	4 473	507 768	UV
15-HOT	8 047	5 312 823	11 640	3 607 668	9 581	4 220	564 381	UV
18-HOT	3 058	3 484 160	2 148	3 390 255	7 351	2 750	497 282	FL
19-COLD	8 461	3 858 924	10 429	3 737 296	10 911	3 345	540 547	
19-HOT	8 850	3 485 163	10 592	3 685 469	9 267	3 356	531 020	
20-HOT	11 053	3 816 468	12 157	3 718 504	11 719	4 651	589 931	UV
21-COLD	9 913	4 035 792	10 130	3 391 752	8 788	4 644	549 656	UV
22-COLD	9 461	4 683 571	10 601	4 358 717	10 417	5 378	596 447	UV
23-COLD	7 395	4 740 654	8 265	3 750 841	7 116	6 282	625 440	UV
23-HOT	8 557	4 436 293	10 390	4 276 659	8 747	4 640	614 484	UV
Number of labs with quantitative values	24	22	23	22	23	21	22	
Mean (IU/kg)	**9 209**	**4 118 352**	**10 845**	**3 861 411**	**9 677**	**4 365**	**566 315**	
Mean (µg/kg)	**2 763**	**1 235 506**	**3 254**	**1 158 423**	**2 903**	**1 310**	**169 895**	
SD_r (IU/kg)	569	206 045	550	174 653	1 221	409	32 798	
RSD_r (%) (repeatability)	6,18	5,00	5,07	4,52	12,62	9,37	5,79	
SD_R (IU/kg)	1 140	465 118	1 171	365 416	1 999	770	61 611	
RSD_R (%) (reproducibility)	**12,37**	**11,29**	**10,8**	**9,46**	**20,66**	**17,64**	**10,88**	
HORRAT	**0,90**	**2,06**	**0,81**	**1,71**	**1,52**	**1,15**	**1,47**	

Graphical presentation of repeatability versus vitamin A content in IU/kg



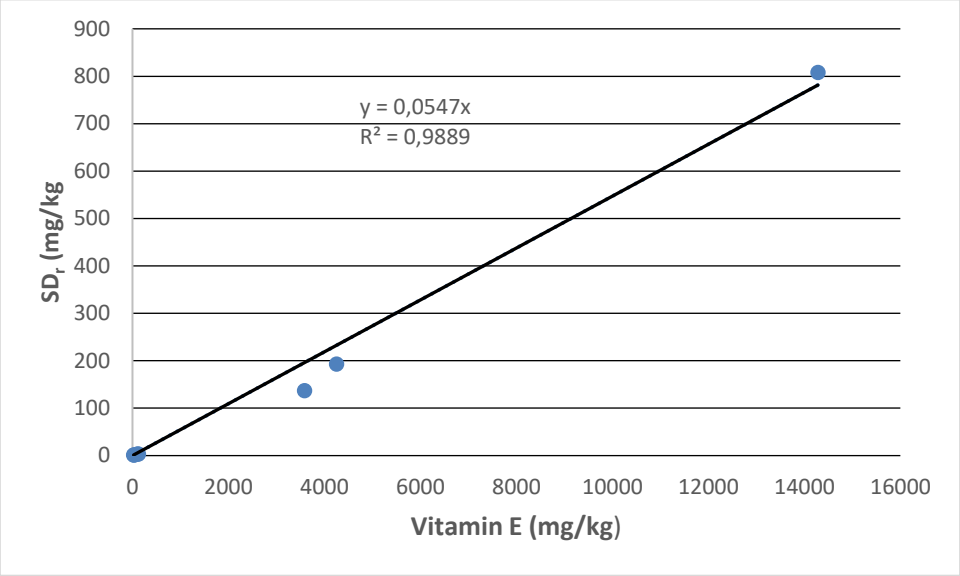
Graphical presentation of reproducibility versus vitamin A content in IU/kg



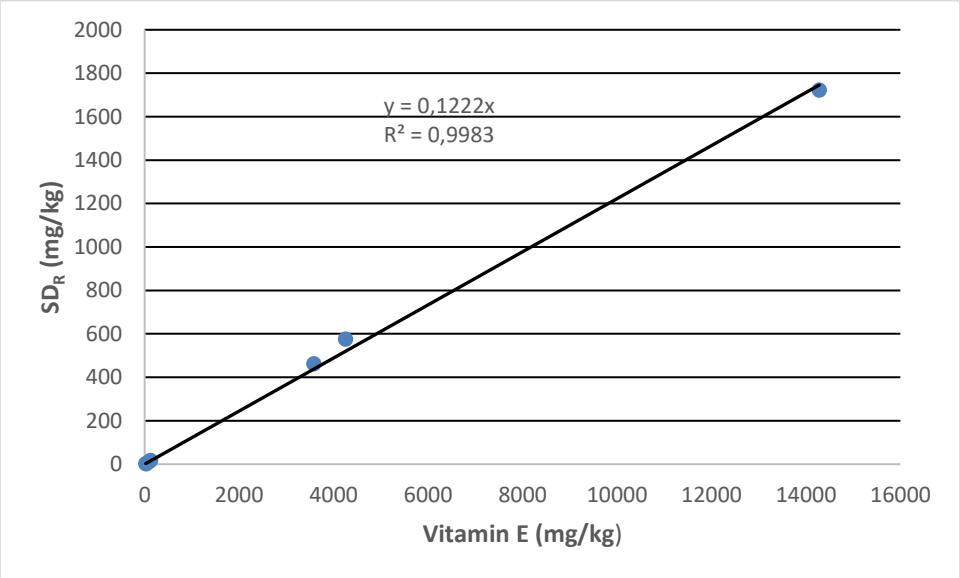
Vitamin E

	1 - KS	2 - PX	3 - KS	4 - PX	5- KS	6- KS	7 - PX	Detection method
Unit	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	
01-HOT	36	4 261	108	15 637	134	24	3 905	FL
~~02-COLD~~	~~19~~	~~2 979~~	~~90~~	~~10 416~~	~~114~~	~~16~~	~~3 269~~	~~UV~~
~~02-HOT~~	~~19~~	~~2 909~~	~~83~~	~~9 831~~	~~102~~	~~18~~	~~2 945~~	~~UV~~
04-COLD	36	5 044	113	15 737	146	27	4 197	UV
04-HOT	39	4 665	114	15 303	128	26	3 987	UV
07-COLD	35							UV
07-HOT	32	4 071	96	11 301	119	23	2 885	UV
09-HOT	34	3 476	99	12 983	115	21	3 158	UV
~~10-COLD~~	~~34~~	~~5 952~~	~~123~~	~~20 341~~	~~171~~	~~24~~	~~5 324~~	-
12-COLD	33	~~3 694~~	100	~~9 469~~	123	23	~~2 761~~	UV
12-HOT	32		98		115	23		UV
13-COLD	27	4 197	86	14 717	120		3 871	FL
13-HOT	28	4 231	88	14 076	118		3 753	FL
14-COLD	32	4 765	106	14 838	100	19	2 884	UV
15-COLD	34	4 035	105	15 769	153	18	4 431	UV
15-HOT	27	5 694	91	16 269	116	18	3 813	UV
18-HOT	35	3 490	113	12 067	117	25	3 024	FL
19-COLD	21	3 885	95	12 734	101	23	2 568	UV
19-HOT	32	4 145	109	14 278	132	24	3 755	UV
20-HOT	32	3 522	79	~~2 857~~	107	22	3 662	UV
21-COLD	33	4 040	97	12 410	108	21	3 470	FL
22-COLD	33	4 516	98	15 491	135	21	3 548	UV
23-COLD	23	4 695	67	14 454	83	34	3 791	UV
23-HOT	35	4 342	101	14 335	123	24	3 589	UV
Number of labs with quantitative values	21	18	20	17	20	18	18	
Mean (mg/kg)	**32,0**	**4250**	**99,0**	**14 282**	**120**	**23,0**	**3 581**	
SD_r (mg/kg)	1,0	193	3,0	808	3,0	1,0	137	
RSD_r (%) (repeatability)	3,25	4,55	3,43	5,66	2,82	4,02	3,8	
SD_R (mg/kg)	3,0	575	12,0	1 723	17,0	3,0	462	
RSD_R (%) (reproducibility)	**10,49**	**13,54**	**12,09**	**12,06**	**14,28**	**14,59**	**12,90**	
HORRAT	**1,10**	**2,98**	**1,51**	**3,18**	**1,84**	**1,46**	**2,76**	

Graphical presentation of repeatability versus vitamin E content in mg/kg



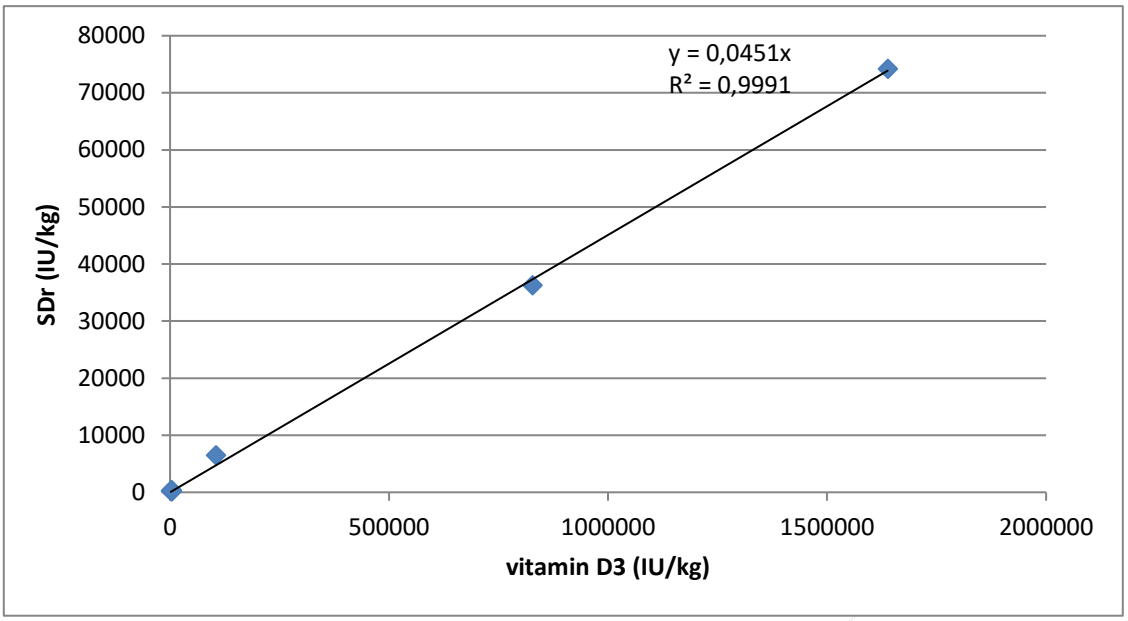
Graphical presentation of reproducibility versus vitamin E content in mg/kg



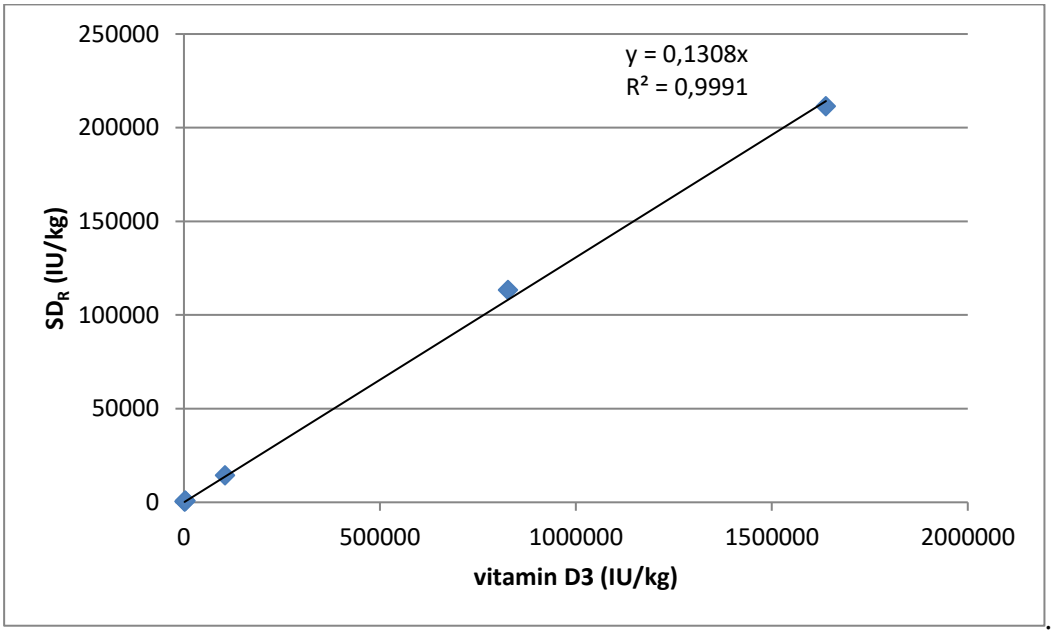
Vitamin D3

	1 - KS	2 - PX	3 - KS	4 - PX	5- KS	6- KS	7 - PX	Detection method
Unit	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	**IU/kg**	
01-HOT	3 432	1 572 501	1 638	920 913	3 747	1 207	99167	UV
02-COLD	3 650	1 465 149	1 708	760 158	5 079	735	100 242	UV
02-HOT	3 482	1 346 362	1 619	724 066	4 048	1 366	88 421	UV
04-COLD	3 597	1 596 667	1 947	824 667	3 447	1 207	97 567	UV
04-HOT	3 620	1 576 667	1 940	838 000	3 057	1 717	95 100	UV
07-COLD	4 979							UV
07-HOT	4 799	1 806 800	2 487	894 033	4 232	2 036	114 233	UV
09-HOT	4 333	1 816 667	2 210	960 000	4 587	2 213	100 333	MS
12-COLD	3 236	2 131 124	2 394	1 004 756	3 367	2 491	119 468	UV
12-HOT	4 128		2 331		3 976	2 159		UV
13-COLD	3 550	1 612 386	2 596	838 394	5 473		100 378	UV
13-HOT	3 815	1 513 939	2 731	838 394	4 111		141 865	UV
15-COLD		1 792 306		866 024			125 355	UV
15-HOT		2 013 051		822 450			127 952	UV
19-COLD	3 120	1 582 187	1 557	700 513	3 000	1 513	86 557	UV
19-HOT	3 273	1 571 847	1 527	672 227	3 047	1 590	100 880	UV
20-HOT	3 639	1 408 083	1 851	747 743	4 295	1 708	89 833	UV
23-HOT								UV
Number of labs with quantitative values	15	15	14	15	14	12	15	
Mean (IU/kg)	**3 772**	**1 638 150**	**2 038**	**826 983**	**3 962**	**1668**	**104 680**	
Mean (µg/kg)	**94**	**40 954**	**51**	**20 675**	**99**	**42**	**2 617**	
SD_r (IU/kg)	200	74 249	242	36 324	411	274	6 524	
RSD_r (%) (repeatability)	5,30	4,53	11,88	4,39	10,37	16,43	6,23	
SD_R (IU/kg)	753	211 463	484	113 457	1 020	572	14 435	
RSD_R (%) (reproducibility)	**19,96**	**12,91**	**23,75**	**13,72**	**25,74**	**34,29**	**13,84**	
HORRAT	**0,91**	**1,41**	**1,08**	**1,35**	**1,17**	**1,56**	**1,00**	

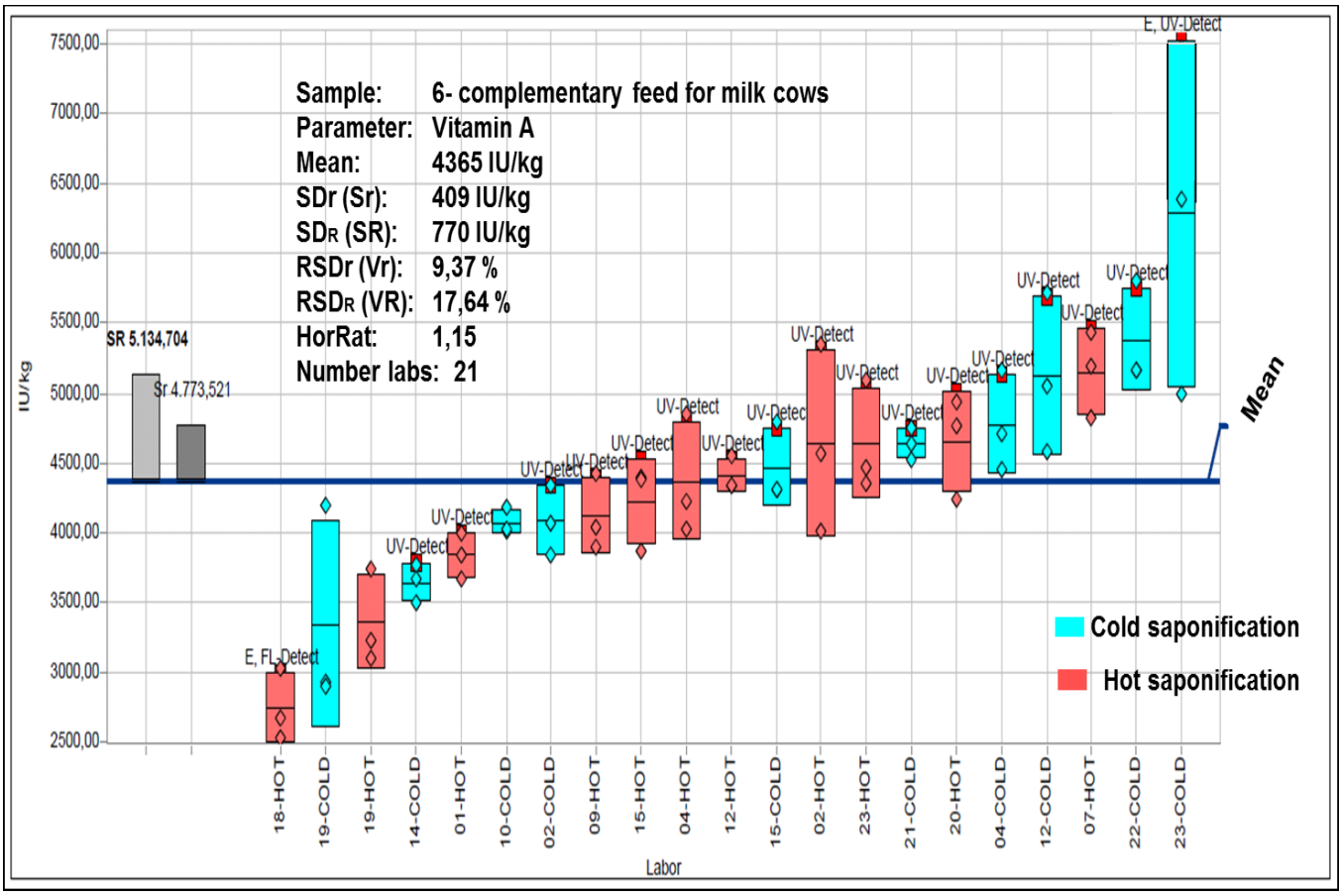
Graphical presentation of repeatability versus vitamin D3 content in IU/kg



Graphical presentation of reproducibility versus vitamin D3 content in IU/kg



Example of graphical presentation of the results of the validation using the example of vitamin A in matrix 6-complementary feed for milk cows



ANNEX 7 – Questionnaire – analytical details

Saponification

Laboratory	Cold saponification	Hot saponification
1		Aliihn-Rückflusskühler
2	Heidolph - Reax 2	IKA - RCT basic safty control
4	Water bath 85 °C for 40 min, Allihn and Dimroth condenser, magnetic stirrer - water bath, self built	In flat bottom flasks on magnetic stirrer over night
5		
9	-	IKA Heating magnetic stirrer, VWR
10	Stirrer REAX 20, Heidolph	
12	Magnitic stirrer, IKA Werke RT 10 Power, IKA	GFL 1042 water bath
13	agitation during about 18 hours (the night for tomorrow)	agitation and boiling during 30 minutes
14	IKA, KS 501 digital	
15	Elpin-Plus S.C. Laboratory shaker type 358A, Multiserv	Multiple water bath 1041, GFL
18		Selecta, water bath
19	Erlenmeyerflask 500 ml (brown) with reflux condenser and tubing for Nitrogen flushing Wisestir SMHS Multiple Point Hotstirrer (2x3 positions)	dtto
20		Water bath magnetic stirrer
21	Over haed shaker (Wagner wheel)	
22	Heidolph Reax 20. Overturning mixer	
23	Roto-Shake Genie, Scientific Industres Inc. (VWR)	Magnetic heater stirrer

Comments

Laboratory	
4	For easier workflow we would suggest solving the BHT in the Ethanol. For easier workflow we would suggest dosing the ascorbic acid with a measuring spoon or spattle.
7	Hot: Heating without water bath; flat bottom flask; Allihn condenser without gas feed pipe
12	The complete and complementary feed samples were weighed 15 g and premixture and mineral samples 5 g into 250 ml flat bottom or conical flasks. The quantities of the saponification reagents were halved but the proportions remained the same. To the complete and complemantary feed was added D2 (4,111 µg/ml) 0,137 - 0,365 ml. To the premixtures and mineral feed was added D2 (205,55 µg/ml) 0,091 - 0,973 ml.
14	Cold saponification was executed in glass erlenmeyers
18	Water bath with Allhin condensers and recirculator/refrigerator connected to condense ethanol vapors. At your instructions there is a section "Samples" where you explained how to prepare the samples, if it's necessary, but because samples did not arrive accompanied by paper instructions (quality department recived them by email) the sample preparation department homogenized and grounded through a sieve of 0.25µm all of them in 02/05/2018
19	Hot saponification measured 13.02.2018 Cold saponification measured 21.02.2018

Extraction

Laboratory	SPE	Trade mark	Total volume
1	CHROMABOND® XTR Polypropylensäulen 70 ml, 14,5mg	Macherey & Nagel	100 ml
2	CHROMABOND® XTR 150 ml, 38 mm	Macherey & Nagel	100 ml
7	CHROMABOND® XTR, Celite, 20 ml	Macherey & Nagel	100 ml
9	CHROMABOND® XTR 70 ml, 14,5 g	Macherey & Nagel	100 ml
4, 10, 14	CHROMABOND® XTR 70 ml,	Macherey & Nagel	70 ml
12	CHROMABOND® XTR, Celite, 30 ml, 20 mm	Macherey & Nagel	~ 85 ml (100-15)
13	CHROMABOND® XTR, 70 ml	Macherey & Nagel	70 ml
15	CHROMABOND® XTR, 70 ml (A,e), 150 ml (D) 30 mm	Macherey & Nagel	80 – 160 ml
18	CHROMABOND® XTR, 70 ml, 30 mm	Macherey & Nagel	Not controled
19	CHROMABOND® XTR, 70 ml 27 mm	Macherey & Nagel	12 ml
20	CHROMABOND® XTR, 70/140 ml	Macherey & Nagel	100 ml
21	CHROMABOND® XTR, 70 ml 25 mm	Merck	160 ml
22	CHROMABOND® XTR, 70 ml 30 mm	Macherey & Nagel	50 ml
23	CHROMABOND® XTR, 70 ml 25 mm	Macherey & Nagel	100 ml

Comments

Lab	
2	Extraction Solvent: Cyclohexane We only use the larger SPE. For samples with low concentrations of vitamin D and after evaporation of the complete extract from SLE the dissolution in 1 ml is difficult since the solution is very viscous. Therefore we dissolved the aliquot in 2 ml n-hexane and the subsuequent vitamin D fraction in 0,5 ml methanol.
4	The volume sum of 130 ml EtOH + 30 ml Na2S + 1 ml EtOH is 157 ml. We did the dilution of decanted 40 ml extract with ascorbic acid solution in a graduated cylinder with a ground neck
10	for control presence of vitamins in column (premix 2PX and 4PX): total results are given in Table 1_OSARK in file Vitamins A+E 02_18_OSARK
12	For the 70 ml SPE columns We pipetted 12 ml of supernatant to the 15 ml graduated flask and filled it with (3 ml) C-vitamin solution and emptied this sample-KOH/Etoh/C-vit-mixture (15 ml) to the SPE column. Then we added cyclohexane until the 100 ml flask became nearly full and filled it up to the volume, 100-15 = 85 ml extraction solvent volume. For the 30 ml SPE columns: Five "cold"samples (2-PX/3, 4PX/2+3, 7PX/2+3) were done by SPEcolumn size 30 ml, since we ran out of the bigger SPEcolumns. We pipetted 4 ml of supernatant to the 5 ml graduated flask and added the rest of the bottle (1 ml) with C-vitamin solution and emptied this sample-KOH/Etoh/C-vit-mixture (5 ml) to the SPE column. Then we added cyclohexane until the 50 ml flask became nearly full and filled it up to the volume, 50-5 = 45 ml extraction solvent volume. Pipe taps were attached to the bottom of the SPE colums to provide a flow of 1 drop/second. * We did not measure the volume of extraction solvent. We added the cyclohexane to the column until the 100 ml flask became nearly full and filled up to the volume.
19	Total volume of extraction solvent: as described in method we take 15 ml of hydrolyse solution. Because this solution is diluted 40/50 it is effectively 12 ml. Or do you mean another volume?
22	Total volume of extraction solvent - complete feed - 50 ml. Total volume of extraction solvent - premixture - 100 ml

Semi-preparative column

Laboratory	Column	Trade mark
1	Si 60, 250x4 mm, 5 µm	Lichrospher Si-60
2	Si 60, 250x10 mm, 5 µm	Merck
4	NP, 250 x 10, 10 µm	Lichrospher, Merck
5		
7	Si 60, 250x4 mm, 5 µm	LiChrosorb, Merck
12	Silica, 300x3,9 mm, 10 µm	uPorasil, Waters
13	LiChrospher 100 Hibar 250x4 mm, 5 µm	Milipore
14	Si 60, 250x8 mm, 5 µm	
15	LiChrospher 100-5 Si, Silica 300 x 8 mm, 5 µm, Merck	Lichrospher
18	No information	
19	Betasil Diol-100, NP. 250x1 mm, 5 µm	Thermo Scientific
20	NP, 25x8 mm, 5 um, MultoHigh 100 Si-5	

Comments

Laboratory	
2	mobile phase 98% n-hexane + 5% iso-propanol (V/V), Agilent 1100 Series, UV detector - VWD Agilent 1100 Series, fraction collector - Agilent 1100 Series
4	We used for the membrane filtration a PET filter with glass fibre, e.g. MN GF/PET-45/25 and we were very pleased with it
9	Vitamin D is detected directly with LC-MS/MS without using a semipreparative clean-up by HPLC
12	Mobile phase: heptane:isopropanol:tetrahydrofuran 98:1:1. Flow rate: 1 ml/min, collection time 3 min, sample is injected 1000 µl and standards 100 µl. The fraction of 3 ml is evaporated. To the sample sis added 150 µl and to the standards 300 µl methanol 90%. Temperature of the column oven is 40 C.
13	The eluent phase used is: n-Heptane 98% / Isopropanol 1% / THF 1%. The retention time is approximately 13 minuts +/- 10%. The lack of some results is explained by the fact that we did not control, at first the collection of fractions; moreover, there were errors on the injected volumes. We replaced hexane with heptane for the recovery of the extract for the semi-preparative chromatography step (2ml and 1ml injected).
15	Samples 1-KS, 3-KS, 5-KS, 6-KS couldn't be processed due to difficulties with dissolving the residue in 1 ml n-hexane. Warming in water nor sonification did not work.
19	We do not do a classic concentration on semi-preparative column. We do 2 dimension LC. We determine the Vitamin D Window on normal phase (NP) and then we switch directly into a loop and further follows a direct injection on RP-Phase column.
20	We use a pre-column 40 x 8 mm with the same material

Standards

Lab	**Standard vitamin A, CAS**	**Standard vitamin E, CAS**	**Std vitamin D3, CAS**	**Std vitamin D2, CAS**	**Supplier**
1	Retinylpalmitat, 79-81-2	Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0		Sigma-Aldrich
2	Retinyl acetate, 127-47-9	DL-α-Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Sigma-Aldrich (A, E), Fluka (D)
4	Retinol 68-26-8	DL-α-Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Sigma-Aldrich, Fluka
5					
7	Retinylpalmitat, 79-81-2	(+/-)-d-Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0		Sigma-Aldrich
9	All-trans Retinol acetate, 127-47-9	DL-α-Tocopherol, 10191-41-0	Vitamin D3 67-97-0		Sigma-Aldrich
10	Retinyl acetate, 127-47-9	α-Tocopherol, 10191-41-0	-	-	Sigma-Aldrich
12	Retinol 68-26-8	DL-α-Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Sigma-Aldrich, Calbiochem
13	Retinol 68-26-8	DL-α-Tocopherol, 10191-41-0	Cholecalciferol, 67-97-0		Sigma-Aldrich, Calbiochem
14	*Retinyl acetate, 127-47-9*	*(+/-)-α-Tocopherol, 10191-41-0*	*Cholecalciferol, 67-97-0*		*Sigma-Aldrich*
15	Retinyl acetate, 127-47-9	DL-α-Tocopherol acetate, 7695-91-2	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Sigma-Aldrich
18	Retinol acetate, 127-47-9	α-Tocopherol, 10191-41-0	-	-	Sigma-Aldrich
19	Retinyl acetate, 127-47-9	DL-α-Tocopherol acetate, 7695-91-2	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Sigma-Aldrich
20	Vitamin A-acetat 127-47-9	Vitamin E acetat 5222-20-4	Cholecalciferol, 67-97-0	Ergocalciferol, 50-14-16	Ehrenstorffer
21	Retinylpalmitat, 79-81-2	α-Tocopherol, 10191-41-0			Sigma-Aldrich
22	Retinyl acetate, 127-47-9	(+)-α-Tocopherol acetete 58-95-7			Sigma-Aldrich
23	Retinylpalmitat, 79-81-2	DL-α-Tocopherol acetate, 7695-91-2			Dr Ehrenstorfer GmbH (VWR)

Comments

Lab	
2	Standard vitamin A (Hot) Retinyl acetate R7882 047K07091 Standard vitamin A (Cold) Retinyl acetate R7882, SLBP8515V Standard vitamin E DL-Alpha-Tocopherol T3251 050M1422 Standard vitamin D2 Ergocalciferol 95220 1295340 Standard vitamin D3 Cholecalciferol 95230 1244338
9	The results for vitamin E are calculated as alpha-tocopherol-acetate
14	*UV-Check 9.2.1.3 Vit A 7.5IU/ml: 327nm, 0.3909 ABS => 7.5 IU/ml* *UV-Check 9.2.2.2 Vit E 500µg/ml: 292nm met 0.3753 ABS => 502 µg/ml* *UV-Check 9.2.3.5 Vit D3 400IU/ml: 265nm met 0.4821 ABS => 419 IU/ml*

HPLC determination

Lab.	HPLC	UV detetor	FL detector	Anal. column	Inj. volume (ul)	Flow rate (ml/min)	Temp. (°C)
1	Shimadzu LC20	Shimadzu SPD-M20A	Shimadzu RF2000	Eurospher C18, 250 x 4 mm, 5 µm	20 (A, E) ~~100 (D3)~~	1	30
2	Dionex UltiMate 3000	Dionex VWD-3400RS	-	LiChroCART, 100 RP-18e, 250 x 4 mm, 5µm	~~20;~~ 100	0,5	20
4	LaChrom L 7100	Merck-Hitachi	LaChrom L 7400	A, E - LiChospher 100, RP-18, 250x4 mm, 5 µm D - Superspher 100, RP 18, 250x3, 4 µm, Merck	100 100	1 0,8	RT
7	JASCO	JASCO MD-4010		LiChospher 100, RP-18, 250x3 mm, 5 µm (A, E) Superspher 100, RP-18e, 250x4 mm, 5µm	25; 100	1	40
9	Hitachi Chromaster	Hitachi Chromaster 5430		RP-18, 250x4 mm, 5 µm, Merck	60	0,5 – 2	20
10	Ultimate 3000	Vanquish		Eclipese C18, 150x4,6 mm, 3,5 µm,	20	1,1	RT
12 (A, E)	Agilent 1100 Series	Agilent DAD		Spherisorb ODS2 (C18 end capted), 150x4,6 mm, 3 µm	10	1	RT
12 (D)	Waters, Aliance 2690	Waters, 2487, dual alpha Absorbance Detector		Vydac 201TP, C18, 150x2,1 mm, 5 µm	10	0,2	40
13	Shimadzu, NEXERA X2	Shimadzu, SPD-M30A	Shimadzu, RF-20A XS	Reverse, C18, 250x3 mm, 5 µm, Supelco	50	1	23
14	Agilent 1100 Series	Agilent 1100 Series	Agilent 1100 Series	Lichospher RP-18, 250x4,6 mm, 5 µm	10; 100	1	20
15	Dionex UltiMate 3000	Dionex UltiMate 3000		Bionacom Velocity, C18, 150x4,6 mm, 5 µm, Genore Chromatografia	100; 20	0,5	25
18	Waters, Aluiance 2795		Waters, Aluiance 2495	RP C18, 150x3,9 mm, 4 µm	20	0,5	RT
19 (A, E)	Agilent 1290	Agilent 1290		Lichosorb, RP-18, 250x4 mm, 7 µm	20	1	40
19 (D)	Dionex UltiMate 3000	Dionex UltiMate 3000		Cosmosil Cholester, RP, 150x4 mm, 7 µm	20	1	25
20	Shimadzu	Shimadzu SPD-20AV	Shimadzu, RF-20A XS	Hypurity C18, 150x4,6 mm, 5 µm	20; 100	0,5	23
21	Agilent 1100	Agilent DAD	Agilent FLD	Eclipse XDB-C8, 150x4,6 mm, 5µm	5 (PX); 30 (KS)	0,8	28
22	Agilent 1100	Agilent 1100	Agilent 1100	Kinetex EVO, C18, 150x4,6 mm, 5 µm	20	0,8	25
23	Agilent 1100	Agilent DAD 1260	Agilent FLD	Lichospher RP-18 endcapped, 250x4,6 mm, 5µm	20	2,5	20

Comments

Lab	
2	mobile phase 100% methanol In the beginning we had problems with our HPLC system and we had to switch the HPLC system. Therefore, the time between the hot saponification and the measurement is extended.
4	A, E mobile phase – 100% MeOH D mobile phase – MeOH / H2O / isopropanol / n-hexane // 1000 / 20 / 20 / 5 ml
9	Vitamin D - MS detection Agilent 1260, Sciex API 5500 Qtrap, column Kinetex C18, 150x3 mm, 5 µm, inj. Vol. 10 – 20 ul, flow rate 0,3 ml/min, temp. 25 °C, Vitamin D3: 385.4> 367.2 (Quantifier), 385.4 > 259.3 (Qualifier) Vitamin D2 (ISTD): 397.0 > 379.2 Vitamin D3-d3 (ISTD): 388.4>370.2 Vitamin D2 is used as internal standard for quantification of samples 2 PX, 4 PX, 7 PX (higher contents) Vtamin D3-d3 is used as internal standard for quantification of samples 1 KS, 3 KS, 5 KS, 6 KS (lower contents)
12	A, E-vitam: Mobile phase: methanol 1000 ml + water 20 ml, samples are in 100% methanol. D3-vitam: Mobile phase 90% methanol, samples are in 90% methanol.

Calibration - Standard stock solution

Lab.	Vitamin	Purity	Supplier	weighed mass (mg)	Concentration STD
1	A	>98	Sigma	55,0	6,8 IU/ml
	E	>98	Sigma	50,0	49,510 µg/ml
	D	>98	Sigma	50,0	18721 IU/ml
2	A (IU/kg) hot	$2{,}8x10^6$	Sigma	38,1	35,7 µg/ml
	A (IU/kg) hot	$2{,}8x10^6$	Sigma	35,7	32,8 µg/ml
	E	>96	Sigma	52,3	523 µg/ml
	D2	>98	Fluka	50,8	508 µg/ml
	D3	>98	Fluka	50,8	505 µg/ml
4	A				
	E				
	D				
7	A				
	E				
	D				
9	A	98 %	Sigma	35,95	73,93 IU/ml
	E	99,6 %	Sigma	100,10	1930 µg/ml
	D	-	-	-	-
10	A (USP units/g)	475 – 650	Sigma	101,4	7,762 IU/ml
	E	96 %	Sigma	50,5	41,633 µg/ml
	D	-			
12	A	99,7 %	Sigma	10,8	54 IU/ml
	E	99,1 %	Calbiochem	114,1	1141 µg/ml
	D3	99,3 %	Sigma	10,9	218 IU/ml
	D2	98,7 %	Sigma	11,1	222 IU/ml
13	A	>= 95%	Sigma	0,1004	505,8 IU/ml
	E	99,5 %	Calbiochem	0,1	1088,0 µg/ml
	D2*	100 %	Sigma	0,1088	966,3 IU/ml
	D3*	98,9 %	Sigma	0,1085	950,1 IU/ml
	D2**	100 %	Sigma	0,05607	546,4 IU/ml
	D3**	98,9 %	Sigma	0,04838	503,3 IU/ml

Lab.	Vitamin	Purity	Supplier	weighed mass (mg)	Concentration STD
15	A	99,9 %	Sigma	50,9	110,3 IU/ml – hot 108,7 IU/ml - cold
	E	100,0 %	Sigma	78,1	52,64 µg/ml –hot 68,66 µg/ml -cold
	D2	98,7 %	Sigma	52,3	20783,5 IU/ml
	D3	100,0 %	Sigma	50,2	21442 IU/ml
18	A	100,0 %	Sigma	43,9	80,14 IU/ml
	E	100,0 %	Sigma	60,5	562,28 µg/ml
19	A	100,0 %	Sigma-Aldrich	62,9	629 IU/ml
	E	100,1 %	Sigma-Aldrich	137,3	1373 µg/ml
	D	100,0 %	Sigma-Aldrich	10,5	105 IU/ml
20	A	99,5 %	Ehrenstorfer	65,8	1309,42 IU/ml
	E	98,0 %	Ehrenstorfer	103,0	2018,8 µg/ml
	D2	99 %	Ehrenstorfer	50	99 IU/ml
	D3	100 %	Ehrenstorfer	49,5	99 IU/ml
21	A	1 810 000 IU/g	Sigma-Aldrich	31,1	73,967 v
	E	98,0 %	Sigma-Aldrich	53,6	538,667 µg/ml
22	A		Sigma-Aldrich	201,2	85,69 IU/ml
	E		Sigma-Aldrich	716,1	529,51 µg/ml
23	A	98,1 %	Ehrenstorfer	53,6	72,36 IU/ml
	E	99,6 %	Ehrenstorfer	74,9	58,68 µg/ml

* : for samples 4PX; 2PX; 7PX

* : for samples 3KS; 1KS; 5KS.

Calibration solutions

Lab.	Vitamin A		Vitamin E		Vitamin D3		Vitamin D2	
	IU/ml	Area	µg/ml	Area	IU/ml	Area	IU/mg	Area
1	0,102	3807	1,98	1373	1,404	1527		
	0,272	9305	7,427	7947	2,808	3370		
	1,36	64834	14,853	16157	4,68	5805		
	2,72	156602	29,706	33515	9,36	12014		
	5,44	316481	59,412	677236	23,4	27231		
	16,32	955519			35,1	46631		
					46,8	60230		
	y = 58984,5x-6612,21; r2 = 0,9998		y = 0,00087294x+0,637654; r2 = 0,9999		y = 1297,51x -500,38; r2 = 0,997			
2 cold	0,108	0,222	0,512	0,137	1,018	0,240	0,816	0,173
	0,288	0,590	2,048	0,557	3,054	0,755	2,448	0,529
	0,577	1,217	4,096	1,101	6,108	1,548	4,895	1,093
	1,441	3,169	7,679	2,054	10,179	2,502	8,159	1,762
	2,883	6,231	15,357	4,171	20,359	5,024	16,317	3,494
	5,765	12,341	30,715	8,042	76,345	18,988	40,793	9,080
	17,296	38,145	61,430	16,012	101,79	24,904	61,190	13,506
							81,587	17,709
	y = 2,2053x-0,0888; r2 = 0,9999		y = 0,2602x+0,0525; r2 = 0,9999		y = 0,2463x +0,0378; r2 = 0,9998		y = 0,2187x +0,0074; r2 = 0,9998	
2 hot	0,055	0,106	0,513	0,134	1,028	0,229	0,798	0,175
	0,148	0,282	2,050	0,506	3,085	0,760	2,395	0,544
	0,295	0,574	4,101	1,026	6,171	1,526	4,790	1,084
	0,739*	1,517	7,689	1,932	10,285	2,486	7,983	1,753
	1,477	3,348	15,378	3,972	20,569	4,957	15,965	3,510
	2,954	5,757	30,755	7,833	51,423	12,674	39,913	8,969
	8,862	17,249	61,510	15,567	77,134	18,674	59,87	13,435
					102,85	24,367	79,826	17,542
	y = 1,9403x+0,091; r2 = 0,9992		y = 12534x+0,0074; r2 = 1,0000		y = 0,2386x +0,0554; r2 = 0,9998		y = 0,2215x +0,0201; r2 = 0,9998	

Lab.	Vitamin A		Vitamin E		Vitamin D3		Vitamin D2	
	IU/ml	Area	µg/ml	Area	IU/ml	Area	IU/mg	Area
4	0,276		2,5		0,8			
	6,906		50		5,0			
	27,622		250		30,0			
	r2 = 0,9997		r2 = 0,9999		r2 = 1,0000			
7	0,3	14891	1,848	11551	0,985	6511		
	0,6	33687	3,696	25932	2,955	17036		
	1,5	91743	6,93	51652	5,91	36528		
	3	194310	13,848	113214	9,85	59341		
	6	393772	27,696	234246	19,7	125072		
	18	1121997	55,392	471082	49,25	324681		
					73,88	505327		
	y = 4488,3x+6,757; r2 = 0,99997		y = 8595x-4995,8; r2 = 0,99995		y = 6863,12x-6780,4; r2 = 0,999733			
9	0,10	108884	0,48	35644	10	212000		
	0,26	336584	1,93	148139	20	385000		
	0,52	735919	3,86	351775	40	789000		
	1,29	1924337	7,24	644961	60	1210000		
	2,59	3924364	14,47	1371979	80	1640000		
	5,18	7488212	28,94	2630259	100	1990000		
	15,59	21491602	57,88	5826902	140	2880000		
					200	4080000		
					240	5040000		
					280	5790000		
	y=7,229e-7x+0,0792; r2 = 0,999556		y=9,862e-6x+0,537; r2 = 0,999799		y=0,0185x+0,00594; r2 = 0,9997			
10	0,1164	0,703	0,4163	0,317				
	0,3105	1,853	2,0817	1,022				
	0,621	3,655	4,1633	1,962				
	1,5524	8,918	7,494	3,56				
	3,196	18,364	18,735	9,119				
	6,1637	34,825	31,225	15,08				
	18,363	10,792	60,368	28,947				
	y=5,5164x+0,2985; r2 = 0,9999		y=0,4797x+0,0362; r2 = 1,0000					

Lab.	Vitamin A		Vitamin E		Vitamin D3		Vitamin D2	
	IU/ml	Area	µg/ml	Area	IU/ml	Area	IU/mg	Area
12	0,41907	12,5853	4,564	16,6286				
	1,2521	37,7482	13,692	51,9387				
	4,1907	124,4011	45,64	174,4221				
	8,3814	246,9687	91,28	349,9960				
13	0,337	54724	0,545	5891	1,09	3639	1,0	2893
	1,683	273902	2,182	26662	2,19	7136	2,0	6358
	3,365	541812	5,45	62857	3,28	11327	3,0	10161
	8,413	1369725	10,91	129450	6,56	29294	6,0	26011
	16,826	2717337	21,81	267069	10,9	39759	10,1	35118
	33,653	5507173	54,53	669033	21,9	75830	20,1	67839
					54,64	187904	50,3	165412
					81,98	279850	75,5	249459
					109,28	372290	100,7	334337
	y=161547x-10973,8; r2 = 1,0000		y= 12018,4x-4631,98; r2 = 1,0000		y = 3392,75 x + 1884,1; r2 = 0,99		y = 3392,75 x + 1884,1; r2 = 0,99	
15 cold	0,163	0,3131	0,687	0,1411			1,04	0,1935
	0,435	0,84435	2,746	0,60345			3,12	0,6454
	0,87	1,7797	5,493	1,2336			6,23	1,3309
	2,174	4,52705	10,3	2,2991			10,39	2,0430
	4,348	8,378					20,78	4,5083
							51,95	11,7184
							77,93	18,1100
							103,90	24,9136
	y=1,9605x; r2 = 0,9978		y=0,2233x; r2 = 0,9986				y=0,235x; r2 = 0,9986	
15 hot	0,165	0,3080	0,526	0,09055			1,07	0,2033
	0,441	0,8885	2,106	0,4117			3,22	0,6954
	0,882	1,8228	4,211	0,84035			6,43	1,4111
	2,206	4,3477	7,896	1,58815			10,72	2,1621
	4,412	8,3326					21,44	4,7479
	8,824	17,8769					53,60	12,3284
	26,472	51,5665					80,40	19,0220
							107,20	26,1573
	y=1,9543x; r2 = 0,9997		y=0,2004x; r2 = 0,9997				y=0,239x; r2 = 0,9987	

Lab.	Vitamin A		Vitamin E		Vitamin D3		Vitamin D2	
	IU/ml	**Area**	**µg/ml**	**Area**	**IU/ml**	**Area**	**IU/mg**	**Area**
18	0,12	43160	0,56	1342050				
	0,32	120585	2,25	5439622				
	0,64	239510	4,50	10855330				
	1,2	451770	8,43	21128024				
	3,21	1333370	16,87	42899982				
	6,41	2470630	33,74	79291609				
	19,23	7389679	67,47	163020099				
	$y=3,84\cdot10^5x+1,28\cdot10^4$; r2=0,9998		$y=2,40\cdot10^6x+ 3,38\cdot10^5$; r2=0,9995					
20	0,0067	40222	0,0715	58104	0,347	53174	0,341	39791
	0,0133	83890	0,143	119761	0,694	105398	0,682	79733
	0,0267	162957	0,2146	181922	1,388	205172	1,363	157805
	0,0533	332196	0,2861	247773	2,776	423120	2,727	323469
	0,0799	498176	0,5722	498705	4,164	635244	4,09	478750
	0,1066	651275	1,4305	1209456	5,552	858487	5,453	648912
	0,2133	1332899	2,157	1791885	8,328	1271569	8,18	956599
	0,7999	4845170	2,8609	2378651	11,104	1724473	10,906	1297063
	1,0664	6466841						
	y = 6E+06x+9538; r2 = 0,9999		y=830110x+8086,2; r2 = 0,9999		y=154941x-5756; r2 = 0,9999		y=118515x-210; r2 = 0,9999	
21	0,074	10,827	0,5387	23,727				
KS	0,2959	37,938	2,1547	88,531				
	0,8876	107,485	6,464	256,725				
	1,4793	191,027	10,7733	451,466				
	127,249x-0,196; r2 = 0,9992		41,476x-1,24; r2 = 0,9999					
PX	1,4793	27,784	1,4793	27,784				
	2,9587	59,351	6,9587	59,351				
	8,876	172,627	8,876	172,627				
	17,752	342,732	17,7521	342,732				
	y = 19,3055x +0,549; r2 = 0,9999		y = 6,695*x +1,044; r2 = 0,9999					

Lab.	Vitamin A		Vitamin E		Vitamin D3		Vitamin D2	
	IU/ml	Area	µg/ml	Area	IU/ml	Area	IU/mg	Area
22	0,129	5,948	0,530	4,985				
	0,343	15,97	2,118	20,23				
	,686	32,11	4,236	40,87				
	1,714	81,14	7,943	77,32				
	3,428	163,6	15,885	155,4				
	6,855	328,8	31,771	311,5				
	20,566	992,8	63,541	636,1				
	y=48,295x-1,022; r2 = 1		y=9,995x-1,655; r2 = 0,9999					
23 Hot	0,11	2,43	0,06	1,3602				
	0,29	6,11	0,23	3,7870				
	0,58	11,97	0,47	7,6538				
	1,45	29,62	0,88	13,8710				
	2,89	63,44	1,76	27,9450				
	5,79	130,02	3,52	60,8513				
	17,37	387,35	7,04	123,8755				
	y = 22,353 x – 0,6957; r2 = 0,9999		y = 17,609 x – 0,8299; r2 = 0,9991					
23 Cold	0,09	1,99	0,06	1,6681				
	0,25	5,16	0,23	5,4108				
	0,50	10,60	0,46	11,1248				
	1,26	25,75	0,86	21,0955				
	2,52	16,12	1,72	42,6729				
	5,03	116,85	3,44	88,3906				
	15,10	344,67	6,89	171,8301				
	y = 22,903 x – 0,7393; r2 = 0,9999		y = 25,071 x – 0,0439; r2 = 0,9997					

Comments

Laboratory	
1	1 Standard bei A und E wurde aus der Kalibrierung entfernt
4	We always use 3 standards for routine measurements
9	Calibration Curve: Analyte Area/IS Area versus Analyte Concentration [µg/kg]. The content of Vitamin D3 is determined in the unit µg/kg. Finally the result is converted to the unit IU/kg by devision with the factor 0,025. Concentration (µg/kg) Area Analyte Area/IS Area 250 1090000 0,19450 250 1070000 0,35981 250 1120000 0,70446 250 1130000 1,07080 250 1060000 1,54717 250 1060000 1,87736 250 1100000 2,61818 250 1080000 3,77778 250 1140000 4,42105 250 1120000 5,16964
12	Vitamin A and E standard stock solutions are made from the alcohol form (retinol and tocopherol). None of the standards are hydrolyzed. Retinol stock solution: 10.8mg/200ml meoh = 54 µg/ml. According to UV check=>53,18 µg/ml At this stage Vitamin E µg/ml is the value of tocopherol. In the end of the analysis the results of Vitamin E are converted into tocopherol acetate. In Reg 152/2009 E1%1cm for tocopherol in ethanol (292nm) is 75,8. In this new method it is 74,7. Which value is recommended? Does there exist E1%1cm for tocopherol in methanol in the literature? Calibration standards are done into methanol. Calibration is not used for the determination of vitamin D3. To every sample is added known amount of internal standard D2. The D3 amount is quantificated by the areas of D2 and D3 in the sample by the equation below: (D3 area in the sample * amount of internal standard (µg) *100) / (D2 area in the sample * the weight of the sample (g)) = µg D3/ 100g
18	The concentration of Vitamin A is calculated in UI/ml because the document CEN/EN 275 indicates these units of concentration.
19	Hot saponification: measured 13.02.2018 Cold saponification: measured 21.02.2018
20	The calibration curves were prepared in 2012 and checked monthly for their correctness by quality assurance actions. All curves were for UV-detection.
23	The SOP is somewhat confusing; it is stated to use the standard stock solution and the working solution to build the calibration curves but the indicative values of the concentration given in the table for vitamin E do not match with the concentration values of the respective stock or working standard solution given in the SOP.

Recovery of vitamin D2 (internal standard)

Lab	1 KS Hot	1 KS Cold	2 PX Hot	2 PX Cold	3 KS Hot	3 KS Cold	4 PX Hot	4 PX Cold	5 KS Hot	5 KS Cold	6 KS Hot	6 KS Cold	7 PX Hot	7 PX Cold
1	95,6		101,2		153,0		95,0		91,3		137,9		92,7	
	92,6		90,4		128,1		91,2		119,5		124,4		107,4	
	67,0		93,7		137,2		293,9*		121,4		154,1		101,2	
2	532	117	87	107	601	142	161	107	607	137	447	81	175	114
	236	154	86	109	1094	229	164	113	617	134	431	189	164	117
	620	80	90	110	1102	244	164	114	609	137	451	24	169	118
4	22	95	72	105	101	123	94	104	139	104	112	109	106	115
	72	92	100	92	76	114	93	103	113	109	109	122	115	102
	108	102	98	108	105	106	83	101	85	116	210	115	102	96
7 -	107,4		87,0		161		80,9		128		147,1		86,3	
	105,6		89,0		174		79,8		132		104,3		89,8	
	103,5		84,0		175		77,8		123		105,0		89,0	
13	86,7	91,4	69,8	93,4	NOT EXPLOITABLE	587	NOT EXPLOITABLE 112,0	No signal	99,4	97,1			INTERFERENCE	
	86,4	88,3	79,6	92,5		77,4		No signal	89,1	100,6			100,0	Too high
	79,6	78,8	86,4	97,3		27,4		98,7	84,1	109,0			80,0	
15	-	-	116,9	103,9	-	-	92,8	99,6	-	-	-	-	112,2	108,2
	-	-	114,5	112,1	-	-	95,8	101,3	-	-	-	-	111,8	110,6
	-	-	109,2	116,5	-	-	96,6	105,0	-	-	-	-	107,9	109,9
19 RF	0,99602	1,00769	0,99602	1,00769	0,99602	1,00769	0,99602	1,00769	0,99602	1,00769	0,99602	1,00769	0,99602	1,00769
20	107,2		94,0		113,1		90,4		80,0		72,7		96,0	
	107,2		91,4		98,8		96,6		75,3		76,1		105,6	
	10,3,6		96,3		96,3		92,1		84,7		60,0		102,4	

Comments

Lab	
1	* 1 KS und 3 KS: Bei der D3-Vorreinigung gab es Probleme - beim Rücklösen in n-Hexan flockte die Probe gelatineartig aus und musste über einen Filter (RC, 0,2µm) gereinigt werden
2	Since the determined recovery in the samples is inconstistent, we determined additional the recovery of vitamin D2 standard: 1) diluted in graduated flask: 100% 2) diluted / evaporated as in Method, without SLE and without fractioning: 83% 3) diluted / evaporated as in Method, without SLE but with fractioning: 76%
9	The quantification is executed by the method of Internal Standard (see worksheed "Calibration Vitamin D"). Therefore a further recovery correction is not necessary.

Lab	
13 – cold	4 PX: Strong dispersion (values D3 and recovery D2) between the three trials. 7 PX first assay: Bad collection on semi preparative chromatography step. 3 KS: Nowadays no explanation about the bad recovery on the first essay and the second essay but our three values on external calibration are good.
13 - hot	7 PX (TOO HIGH): addition D2 after purification by SPE. 4 PX (NO SIGNAL HPLC): Because vitamin D not collected by semi preparative chromatography step. 3 KS: Mistake about metered addition very probable.
19	we calculate the response factor, the ratio between Vitamin D2 and D3 therefore we use Vitamin D2 as internal Standard for the determination of D3. All samples are corrected with this factor. The reason is that we do not collect the Vitamin D fraction in normal phase, we inject directly by loop from normal phase into reversed phase.

ANNEX 8 – Bibliography

EN ISO 14565 Animal feeding stuffs — Determination of vitamin A content —Method using high-performance liquid chromatography

EN ISO 6867 Animal feeding stuffs — Determination of vitamin E content —Method using high-performance liquid chromatography

Commission Regulation (EC) No 152/2009 of 27 January 2009, laying down the methods of sampling and analysis for the official control of feed, Annex IV/A and ANNEX IV/B

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EN 12822 — Foodstuffs – Determination of vitamin E by high performance liquid chromatography: Measurement of α-, β-, γ- and σ- Tocopherol

EN 12823-1 — Foodstuffs – Determination of vitamin A by high performance liquid chromatography – Part 1: Measurement of all-trans-retinol and 13-cis retinol

ISO 5725-1 — Accuracy (trueness and precision of measurement methods and results – Part 1: General principles and definitions

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