RIKILT
WAGENINGEN UR

Animal feedingstuffs - determination of hydrocyanic acid by HPLC: results of the collaborative study

Mandate 382 to CEN/TC 327

RIKILT Report 2011.001

W.M.J. Beek and J. de Jong

Animal feedingstuffs - determination of hydrocyanic acid by HPLC: results of the collaborative study

Mandate 382 to CEN/TC 327

W.M.J. Beek and J. de Jong

Report 2011.001 **June 2011**

Project number: 120.72.52.401
Project title: NEN hydrocyanic acid

Project leader: W.M.J. Beek

RIKILT - Institute of Food Safety
Wageningen UR (University & Research centre)
Akkermaalsbos 2, 6708 WB Wageningen, The Netherlands
P.O. Box 230, 6700 AE Wageningen, The Netherlands
Tel. +31 317 480 256
Internet: www.rikilt.wur.nl

This research was funded by the European Commission under the grant agreement SA/CEN/ENTR/382/2007-03 to CEN/NEN.

Distribution list:

- AGES - Agency for Food and Health Safety (Vienna, Austria); I. Strnad
- Bavarian Health and Food Safety Authority (Oberschleissheim, Germany); C. Schlicht
- Chemical and Veterinary control laboratory Münster (Münster, Germany); T. Bernsmann
- CVUA Sigmaringen (Sigmaringen, Germany); G. Thielert
- Joint Research Centre, IRMM (Geel, Belgium); J. Stroka and A. Breidbach
- Laboratorio Arbitral Agroalimentario (Madrid, Spain); J. Munoz
- LAREAL (Saint Nolff, France); R. Le Bouquin
- LTZ Augustenberg (Karlsruhe, Germany); A. Trenkle
- LUFA Speyer (Speyer, Germany); J. Kühl and P. Dieterle
- Minton Treharne & Davies Ltd (Cardiff, United Kingdom); J. Robinson
- Minton Treharne & Davies Ltd (Llanelli, United Kingdom); M. Dalling and H. Evans
- National Reference Laboratory (Budapest, Hungary); J. Dömsödi
- Oleotest (Antwerpen, Belgium); P. Bastijns
- SCL-Laboratoire de Rennes (Rennes, France); A. Cristol
- The Danish Plant Directorate (Lyngby, Denmark); A. Plöger
- TUM, Bioanalytik (Freising, Germany); J. Darnier
- West Yorkshire Analytical Services (Leeds, United Kingdom); D. Campbell
- Worchester Scientific Services (Worchestershire, United Kingdom); E. Moran
- Wageningen UR (Wageningen, The Netherlands); W. Traag. A. Jongbloed and J. Thissen
- European Commission, DG Health and Consumer Protection (Brussels, Belgium); F. Verstraete
- Netherlands Standardization Institute - NEN (Delft, the Netherlands); F. van der Zweep
- RIKILT - Institute of Food Safety (Wageningen, the Netherlands); A. Koot, J. Kiers, M. Alewijn and S. van Ruth

Summary

A method is proposed for the determination of Hydrocyanic acid (HCN) in animal feed and feed materials of plant origin at concentration levels fulfilling the requirements of EC-Directive 2002/32, describing the maximum allowed concentration of hydrocyanic acid in feed and feed materials.

The principle of the method is extraction of cyanoglycosides from feed, followed by acid incubation and enzymatic breakdown to cyanide. Isolation of the hydrocyanic acid from the sample by steam distillation, and then derivatisation with taurine and NDA (2,3 napthylene dicarboxy aldehyde) to form a fluorescence complex, and determination by HPLC with fluorescence detection.

The performance characteristics of this analytical method were determined via a collaborative study. The relative standard deviation found for repeatability (RSD_r) ranged from 1.4 - 10.4% and the corresponding relative standard deviation of reproducibility (RSD_R) ranged from 7.2 - 38.8%. In general, the $HORRAT_R$ values were equal to or below the critical value of 2. One out of eight positive samples the $HORRAT_R$ value exceeded 2, viz. 3.3 for a chicken feed with a mean content of 7.7 mg/kg. For spiked feed samples at the level of 10 and 50 mg HCN/kg the average value for recovery was 63% and 70%, respectively.

When correction for recovery rate is applied, the relative standard deviation for repeatability (RSD_r) ranged from 2.6 - 11.4% and the corresponding relative standard deviation of reproducibility (RSD_R) ranged from 7.8 - 22.8%. Only for one out of six positive samples the $HORRAT_R$ value slightly exceeded the value of 2, viz. 2.1 for a chicken feed with a mean content of 12.5 mg/kg.

Recovery correction leads to improved performance characteristics of the method with regards to $HORRAT_R$ values and number of outliers. While recovery correction has to be applied because of EC-legislation, the recovery corrected figures for the performance characteristics should be included in the draft CEN-method.

The method proved to be applicable to analyse cyanide in various feeds and feed materials, viz. chicken feed, pig feed, horse feed, tapioca, linseed and almonds. The method is applicable in the concentration range of 2 - 400 mg/kg. The limit of quantification of the method is 2 mg HCN/kg feed.

Consequently, it was concluded that the method is suitable for quantitative analysis. The proposed method is able to detect hydrocyanic acid at the limits according to European legislation viz. 10 mg HCN/kg for chicken feed and higher contents for other feeds and feed materials.

Contents

1 Introduction

Cyanide is a highly toxic compound; in feed it is mainly bound as cyanoglycosides. Cyanide is released from cyanoglycosides by enzymatic breakdown, which process is known as cyanogenesis. So far, the method as formerly described in First Commission Directive 71/250/EEC was used in the EC as a reference method to determine the content of cyanide (expressed as hydrocyanic acid) in animal feed and feed materials. However, the reference method proved to be not sufficiently sensitive and robust. According to EC-directive 2002/32, detection at a low level of 10 mg HCN/kg should be possible. However, the method does not fulfil this requirement. In addition, thiocyanates are detected as well and false positive results could be obtained. For these reasons this method was deleted from EC legislation (Commission of the European Communities, 2009, Reg. (EC) No. 152/2009).

To overcome these disadvantages an improved method has been developed at RIKILT which is more reliable, sensitive and robust. The former EC protocol consists of 4 steps: 1) extraction of cyanoglycosides, 2) formation of hydrogen cyanide by enzymatic degradation, 3) extraction of hydrogen cyanide by steam distillation, and 4) titrimetric detection of cyanide. The first two steps were improved by using a weak acid for extraction of cyanoglycosides (step 1) and applying commercially available enzyme instead of an almond suspension (step 2). Instead of the titrimetric method (step 4), cyanide detection was performed by derivatization with taurine and NDA (2,3 napthylene dicarboxy aldehyde), to form a fluorescence complex, and determination by HPLC with fluorescence detection. The detection of the cyanide-complex is selective and not interfered by other compounds.

The method has been selected for standardization by CEN (European Committee for Standardization) in the committee CEN/TC 327 Animal feedingstuffs - Methods of sampling and analysis. The method was initially investigated in a pre-trial by four European laboratories during autumn 2008. The transferability of the method proved to be possible, but further improvement of the robustness was needed. The results of the pre-trial were discussed with the participants and suggestions for improvement of the robustness were made. Further experimental work was done at RIKILT, leading to an improved protocol that was deemed ready for collaborative study of the method. In June 2009, the international collaborative study started. This paper reports the results of the collaborative study to validate the HPLC method for the detection of hydrocyanic acid in feed and feed materials.

2 Methods and materials

2.1 Test Materials for the Collaborative Study

For this inter-laboratory study products were provided by a local animal feed warehouse, by dr. Gerhart Thielert, CVUA Sigmaringen, Germany, dr. Age Jongbloed and mr. Wim Traag, Wageningen UR, Wageningen, the Netherlands.

The test materials were compound feedstuffs and feed materials containing typical ingredients using a realistic recipe. To obtain naturally HCN-contaminated test materials, the feeds and feed materials were mixed with highly contaminated linseed to achieve the desired levels. The materials were subsequently filled into containers (about 40 grams each container) and stored at 4-8 °C. The containers were kept at this temperature until analysis for homogeneity and dispatch for collaborative testing.

The following samples have been prepared: blank chicken feed, chicken feed, pig feed, horse feed, tapioca, linseed and almonds.

2.2 Homogeneity of test materials

The test samples were analysed for homogeneity using the HPLC-FI method.

Ten (or nine) containers of the materials were analysed in duplicate. The homogeneity study was carried out according to 'The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories' (1) and ISO/DIS 13528 (2), taking into account the insights discussed by Fearn et al. (3) and Thompson (4).

Simultaneous with the analysis of each of the materials, at least two samples of the blank feed were analysed. Those analyses demonstrated that the material was free of Hydrocyanic acid (<0.5 mg/kg) and is therefore suited to use as a blank material in the inter-laboratory study.

An overview of the results from the analyses is shown in Table 1. The figures are not corrected for recovery. All materials were sufficiently homogeneous for use in the inter-laboratory studies.

2.3 Organisation of the collaborative study

Prior to the trial, laboratories across the EU received an invitation to participate in the collaborative study. A total of 19 laboratories responded and confirmed to participate. All were invited to participate, without any selection.

For the collaborative study each participant received information about how to carry out the study and to report the results.

Table 1. Results of the analyses on concentration and homogeneity.

Sample type	Id. Nr.	Average concentration (mg HCN/kg)	CV (%) (n=10; 2 replicates)	Homogeneity
Blank feed	RI_AOAI_20	-	-	-
Chicken feed	RI_AOAI_22	8.1	4.2	accepted
Pig feed	RI_AOAI_23	40.4	1.9	accepted
Horse feed	RI_AOAI_24	64.3	1.9	accepted
Tapioca	RI_AOAI_25	94.1	1.2	accepted
Linseed	RI_AOAI_27	240.3	1.3	accepted
Almonds	RI_AOAI_28	44.3	2.9	accepted

2.4 Study design

The set-up and execution of the collaborative study has been done according to the IUPAC protocol (Horwitz, 1995) for the design, conduct and interpretation of method-performance studies.

According to these guidelines ≥5 different samples should be analysed in ≥8 laboratories.

A detailed method protocol, samples for analysis, results forms, specific details on method execution and a questionnaire were sent on June 22, 2009 to each participant.

Table 2 lists the materials used in this study and supplied to collaborative study participants.

Table 2. Samples supplied to collaborative study participants.

Sample type	Id. Nr.	Indicative concentration range (mg HCN/kg)
Blank chicken feed	RI_AOAI_20	-
Chicken feed	RI_AOAI_22	5 - 100
Pig feed	RI_AOAI_23	5 - 100
Horse feed	RI_AOAI_24	5 - 100
Tapioca	RI_AOAI_25	25 - 400
Linseed	RI_AOAI_27	25 - 400
Almonds	RI_AOAI_28	25 - 400
Blank chicken feed	RI_AOAI_20	to be spiked at a concentration of 10 mg and 50 mg HCN/kg

The test samples for the collaborative study were homogenised, grinded and ready to use, preparation of materials (chapter 6 of method procedure) is not necessary. The amount of feed in each sample bottle is about 40 grams. For a single analysis 5 grams of sample material is needed.

It was asked to analyse all the feed and feed material samples in duplicate, plus spike samples (in duplicate).

The labs were asked to note the found content in an accompanying table, to fill out a questionnaire and to return it before September 15, 2009.

The labs that actually took part have received a fee, as a contribution to material costs for carrying out the experiments.

2.5 Method of analysis

Principle of the method: Extraction of cyanoglycosides from feed, followed by acid incubation and enzymatic breakdown to cyanide. Isolation of the cyanide from the sample by steam distillation, and then derivatisation with taurine and NDA to a fluorescence complex. Analysing the complex by HPLC with fluorescence detection. The limit of determination of the method, as determined at RIKILT during in-house validation, is 2 mg HCN/kg feed.

3 Results and discussion

3.1 Pre-collaborative study

A pre-trial has been organized aiming to study the performance of the method in other laboratories (transferability of the method). A total of 4 of 5 invited laboratories reported results.

All the laboratories analysed the samples - blank feed, feed 5-10 mg HCN/kg, feed 10-50 mg HCN/kg and linseed 50-500 mg HCN/kg - in duplicate, the results were submitted without corrections for recovery.

For linseed a satisfactory reproducibility was obtained. For the feed samples the reproducibility was unsatisfactory, due to the fact that two of the four labs obtained lower concentrations.

For this reason the robustness of the method has been further improved by RIKILT, taking into account the recommendations/remarks of the pre-trial study participants.

In particular, the sample preparation procedure has been further studied. While hydrocyanic acid is volatile at acidic conditions at temperatures of 26 °C and higher it is important to use screw-capped bottles and cooled solutions in the relevant parts of the protocol. Other modifications included increasing the concentration of the enzyme to be added and addition of EDTA to complex Fe++ ions in order to prevent formation of hexacyanoferrate.

The method description has been adapted accordingly.

3.2 Collaborative study

19 laboratories across Europe indicated to participate in the collaborative study. All laboratories were accepted, without any selection, to participate in the study. In total, 17 laboratories delivered results. However, one laboratory (lab nr. 2) deviated significantly from the method description and consequently the results were not considered valid. The sample (at 8.3.1) was weighted directly into the steam-distillation bottles, instead of weighing in a screw cap bottle. However, the sample had to be weighed into screw-cap bottles because normally, the distillation bottom-flasks cannot be closed in an appropriate way, which may lead to loss of hydrocyanic acid. The approach followed by lab 2 may lead to satisfactory results, but this has to be investigated further.

The data sets of the remaining 16 laboratories (see Table 3) were considered as valid, and further evaluated and included for statistical analysis.

3.3 Statistical analysis of results

Introduction: The study design and target performance characteristics for the evaluation of the LC-Fl method were selected according to internationally accepted guidelines for method validation (ISO 7525, Horwitz 1995). Quantitative results submitted by the laboratories were used to estimate average concentrations and standard deviations under repeatability and reproducibility conditions.

Results are subjected to a graphical consistency technique, viz. the Mandel h and k statistics (ISO 7525) to give insight in the overall performance for each of the participating laboratories. The Mandel h plot shows the between-laboratory consistency statistic and the Mandel k plot shows the within- laboratory consistency statistics. As a second step, the valid data must be purged of all outliers flagged by the harmonized outlier removal procedure. This procedure consists of the sequential application of the Cochran and single and double Grubbs test (at 2.5% probability level, one-tail for Cochran and two tails for Grubbs) until no further outliers are flagged or until drop of 22.2% (=2/9) in the original number of laboratories providing valid data occurred.

The precision data obtained from the collaborative study were then compared with predicted acceptable levels of precision. These levels, as estimated by the Horwitz equation (Horwitz et al., 1980), provide an indication as to whether the method is sufficiently precise for the concentration level of the analyte being measured, which is expressed by the HORRAT value (Horwitz and Albert, 2006). For between-laboratories reproducibility, the HORRAT value compares the measured reproducibility with the precision calculated by the Horwitz equation at that particular analyte concentration level: $HORRAT_R = RSD_R/RSD_{R,Horwitz}$.

RSD_R is the relative standard deviation of reproducibility obtained in the validation exercise and $RSD_{R,Horwitz}$ is the calculated stand deviation of reproducibility using the Horwitz equation:

$RSD_{R,Horwitz} = 2^{(1-0.5\log C)}$, where C is the mass concentration expressed to the power of 10 (i.e. 1 mg g^{-1} = 10^{-3}).

The target value for the HORRAT value is set at 1; the rejection level is 2.

3.4 Evaluation of the results (without recovery correction)

The Mandel h and k plots are shown in Figure 1. The Mandel h plot shows that the majority of the values of lab 1 are extreme: lab 1 is an outlier for 4 (1% significance level) or even 5 (5% significance level) out of the 8 positive samples, including the 2 recovery samples. For this reason all the results of lab 1 have been removed. Lab 1 was contacted to enquire about possible reasons for the extreme values. They replied that no reason could be found.

The results of the Grubbs and Cochran outlier tests performed on the results of the 15 labs are presented in Table 3. For linseed, next to lab 1, two other outliers were excluded, viz. lab 5 (Grubbs upper outlier) and lab 18 (Grubbs double lower outlier). In fact lab 12 was also a Grubbs double lower outlier but this result has not been excluded because a maximum of 22.2% of the original number of labs can be excluded.

The results of the statistical analysis, including the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories and the $HORRAT_R$ ratio are presented in Table 4.

The RDS_r range is from 1.4 - 10.4% and the corresponding RDS_R range from 7.2 - 38.8%. The $HORRAT_R$ values range from 1.1 - 3.3 with one value exceeding 2, viz. 3.3 for the chicken feed with a mean content of 7.7 mg/kg.

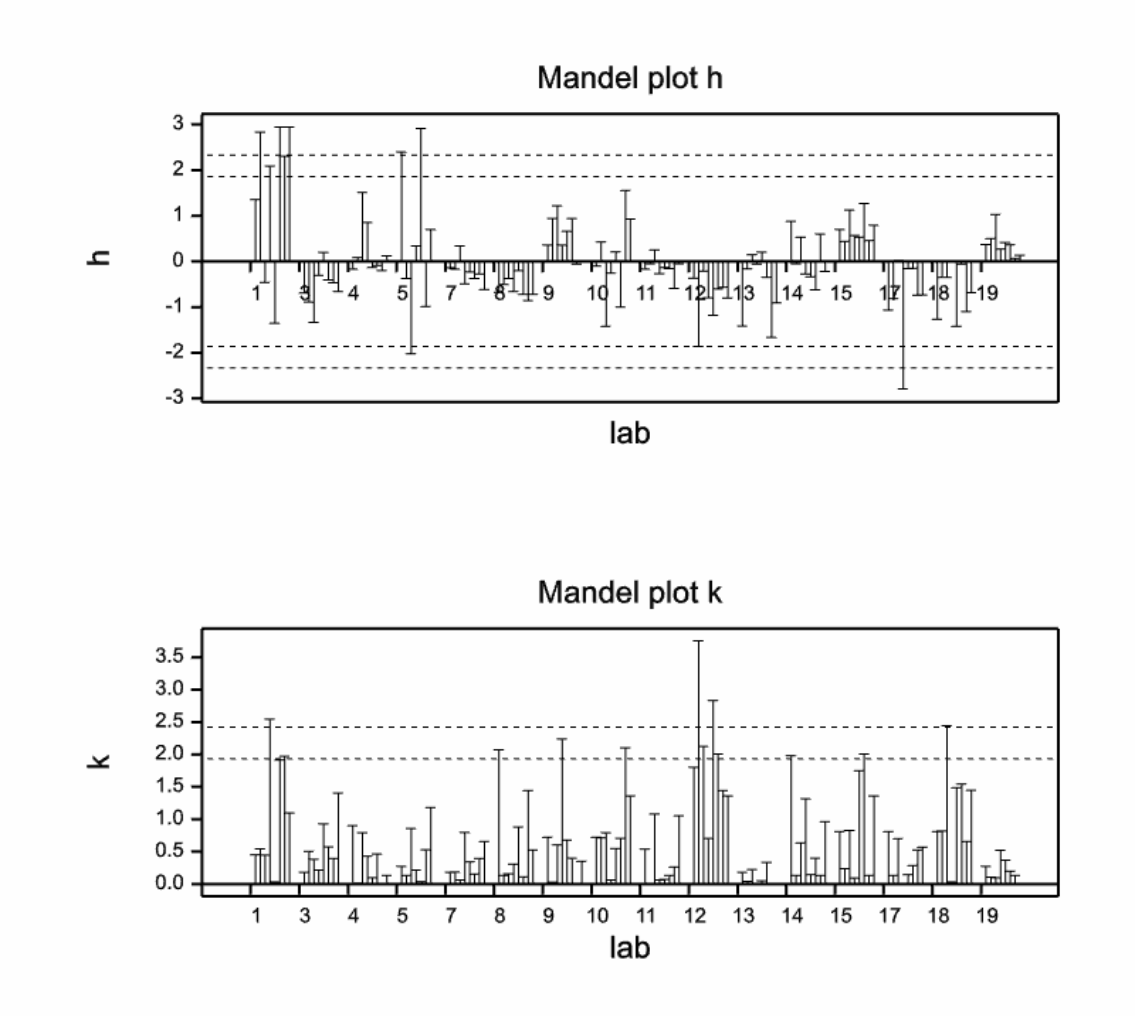

Figure 1. Mandel h and k plots; the dashed lines are the indicator lines for 1 (upper dashed line) and 5% (lower dashed line) significance levels.

3.5 Evaluation of the results after recovery correction

For spiked feed samples at the level of 10 and 50 mg HCN/kg the average value for recovery was 63% and 70%, respectively (expressed as HCN concentration, see Table 4). For recoveries below a recovery rate of 90% the HCN-concentration found in naturally contaminated materials should be corrected (Commission of the European Communities, 2009, Reg. (EC) No. 152/2009). This correction has been applied on the reported figures of the collaborative study. For each lab, the figures at a level below 25 mg HCN/kg feed (chicken feed) have been corrected using the respective value for recovery found by this lab at the spike level of 10 mg/kg. The figures at a level above 25 mg/kg (pig feed, horse feed, tapioca, linseed and almonds) have been corrected using the respective value for recovery found by this lab at the spike level of 50 mg/kg. An overview of the individual results, corrected for recovery, is given in Table 5.

These results after recovery correction are also subjected to graphical consistency technique, viz. the Mandel h and k statistics to give insight in the overall performance for each of the participating laboratories. An overview is shown in Figure 2.

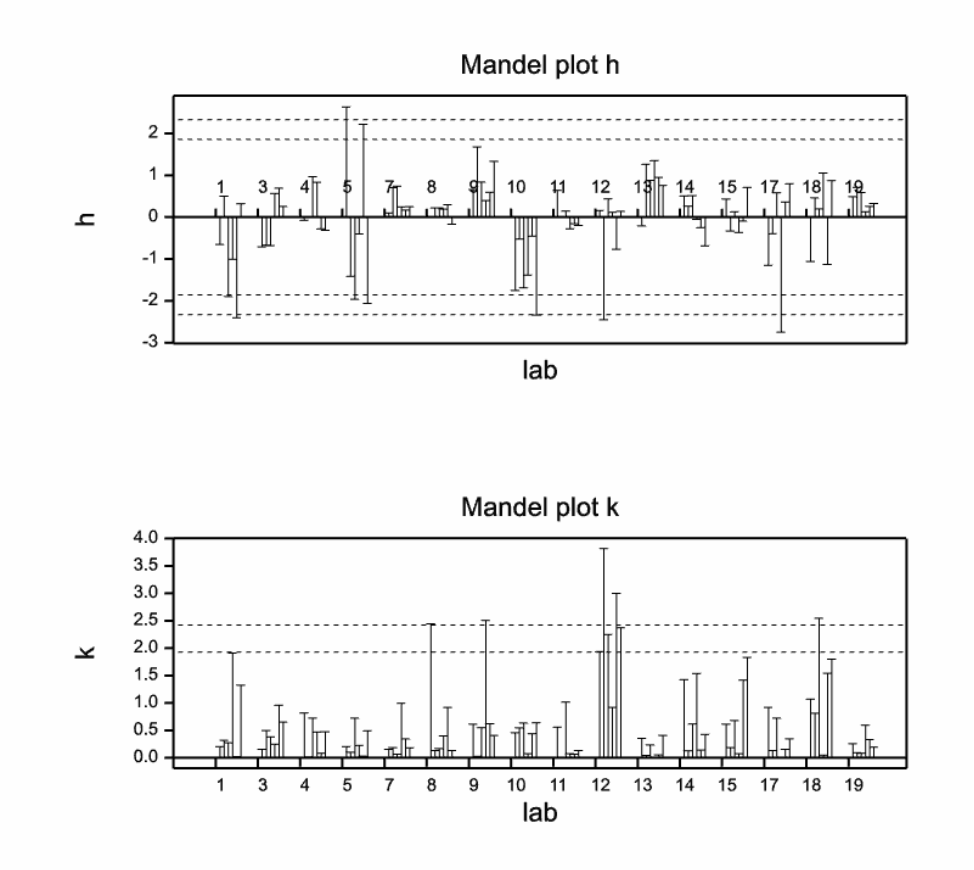

Figure 2. Mandel h and k plots of the results after recovery correction; the dashed lines are the indicator lines for 1 and 5% significance levels.

The Mandel h plot shows that the values of none of the labs are extreme, consequently no labs have been removed. However, the results of lab 13 were excluded for further evaluation because recovery spikes were single values reported instead of duplicate values. For lab 5 the chicken feed results have been corrected using the respective values for recovery found by this lab at the spike level of 10 mg/kg. The figures at a level above 25 mg/kg (pig feed, horse feed, tapioca, linseed and almonds) could not be corrected because no values for recovery were reported at the spike level of 50 mg/kg.

The results of the Grubbs and Cochran outlier tests performed on the results of the labs are presented in Table 5.

The results of the statistical analysis of the recovery corrected figures, including the average analyte concentration, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories and the $HORRAT_R$ ratio are presented in Table 6.

The RDS_r range from 2.6 - 11.4% and the corresponding RDS_R range from 7.8 - 22.8%. The $HORRAT_R$ values for between-laboratories reproducibility range, from 0.9 - 2.1 with 1 value exceeding 2, viz. 2.1 for the chicken feed with a mean content of 12.5 mg/kg.

3.6 Discussion of the statistical results

Most $HORRAT_R$ values were equal to or below the critical value of 2. At concentrations in the lower range of 7.7 (12.5 corrected for recovery) (near LOQ) the $HORRAT_R$ did not meet the target value of 2, the value found was 3.3 without recovery correction and 2.1 after recovery correction.

For spiked feed samples at the level of 10 and 50 mg HCN/kg the average value for recovery was 63% and 70%, respectively. For recoveries below a recovery rate of 90% the HCN-concentration found in naturally contaminated materials should be corrected (Commission of the European Communities, 2009, Reg. (EC) No. 152/2009) This correction has been applied on the reported figures of the collaborative study.

The results clearly show that recovery correction leads to better performance characteristics of the method. In general, lower $HORRAT_R$ values are obtained and less outliers have to be removed. Moreover, the results of lab 1, that had to be removed when recovery correction was not applied due to many extreme values (Mandel h graphical consistency technique), could be taken into account after recovery correction.

The improvement of results after recovery correction shows that the difference in recovery is one of the main reasons for the variability between laboratories. This difference in recovery is caused by the loss of HCN during enzymatic breakdown at acidic conditions and the transfer of the solution into the steam distillation flask, which can be regarded as the most critical step in the method. Nevertheless, the results of the statistical evaluation after recovery correction show that the method is fit for the purpose.

The results of lab 2, which were not included in the statistical evaluation because they did not use the prescribed method of analysis, show compliance with the results of the evaluated labs after recovery correction (see Table 7).

Only at concentrations in the lower range of 12.5 mg HCN/kg the $HORRAT_R$ did not meet the target value of 2, the value found was slightly above 2, viz. 2.1.

Table 3. Overview individual results (in mg HCN/kg) and outliers.

Lab code	Blank feed		Chicken feed		Pig feed		Horse feed		Tapioca		Linseed		Almonds		Blank feed spike 10 mg/kg		Blank feed spike 50 mg/kg	
1	<1[a]	<1[a]	12.4[a]	11.9[a]	63.1[a]	59.0[a]	53.0[a]	54.4[a]	116.2[a]	107. 9[a]	153.3[a]	152.7[a]	68.5[a]	59.8[a]	10.5[a]	12.0[a]	54.9[a]	52.4[a]
3	0.3	0.2	5.8	6.0	30.2	34.0	44.8	46.0	81.5	82.2	224.0	242.6	39.5	36.9	5.4	5.7	30.6	33.8
4	0.2	0.2	8.0	7.0	39.7	39.6	71.2	73.7	97.2	95.8	217.0	215.1	41.7	39.6	6.1	6.1	36.7	37.0
5	3.8	3.0	15.2	15.5	36.6	35.6	37.5	40.2	89.6	90.3	***373.4***	***374.1***	32.5	34.9	7.5	8.4	-	-
7	0.0	0.0	7.5	7.7	38.4	37.0	61.2	61.4	80.8	78.2	207.6	214.4	38.1	38.8	6.1	5.8	31.7	33.2
8	0.3	0.4	7.1	4.8	34.6	35.6	54.8	54.3	77.9	76.9	203.8	221.4	35.5	36.0	4.2	5.3	31.2	32.4
9	0.2	0.6	9.5	8.7	46.2	46.4	68.7	70.6	**86.6**	**93.9**	250.4	264.0	49.5	47.7	6.4	6.4	35.8	36.6
10	0.8	2.0	8.1	7.3	39.6	45.0	43.3	45.8	82.4	82.6	239.3	228.3	35.2	32.0	8.9	10.5	43.2	40.1
11	0.0	0.0	7.8	7.2	38.6	38.6	58.8	62.2	82.4	82.2	215.6	217.0	39.8	40.4	5.4	5.2	37.0	34.6
12	0.0	0.0	5.9	7.9	**38.8**	**10.3**	59.4	52.7	74.5	76.8	133.7	190.5	41.2	32.1	5.9	4.8	32.9	29.8
13	0.1	0.1	3.8	3.6	37.6	37.9	59.1	59.8	85.0	85.0	233.0	234.0	39.4	37.9	3.1[b]	-	30.7[b]	-
14	0.2	0.2	11.8	9.6	39.1	38.1	64.1	62.1	80.1	84.4	207.1	204.2	37.4	35.6	7.8	7.7	35.9	33.7
15	0.2	0.2	10.6	9.7	43.3	41.5	70.1	67.5	93.0	92.7	268.0	233.0	55.7	46.6	7.4	7.5	39.3	42.4
17	0.0	0.0	5.2	4.3	32.2	33.2	57.2	59.4	50.4[b]	-	216.4	213.5	40.8	39.5	5.2	4.8	32.4	31.1
18	0	0	4.6	3.7	39.4	33.2	58.6	50.9	86.0	85.9	***164.3***	***134.5***	44.4	37.4	4.5	4.0	33.7	30.4
19	<1	<1	9.0	9.3	42.5	43.3	67.7	68.0	90.0	88.3	240.8	248.2	43.7	44.6	6.7	6.6	36.9	36.9

*Outliers identified by Cochran (**bold**) and Grubbs (**italics, bold**).*
Outliers identified by operator: [a] Mandel h outliers (all results of lab 1); [b] single values.

Table 4. Results statistical analysis.

	Chicken feed	Pig feed	Horse feed	Tapioca	Linseed	Almonds	Feed 10 ppm	Feed 50 ppm
Mean (mg/kg)	7.7	38.5	58.4	84.7	227.4	39.9	6.3	35.0
N	16	16	16	15	16	16	15	14
Outliers	1	2	1	2	3	1	1	1
S_r (mg/kg)	0.8	1.8	2.3	1.2	14.6	2.9	0.5	1.6
S_R (mg/kg)	3.0	4.2	9.9	6.1	26.5	5.4	1.5	3.7
RSD_r (%)	10.4	4.7	3.9	1.4	6.6	7.2	7.6	4.6
RSD_R (%)	38.8	10.9	17.0	7.2	11.9	13.4	24.2	10.5
r (mg/kg)	2.3	5.1	6.4	3.4	40.9	8.0	1.3	4.5
R (mg/kg)	8.4	11.8	27.7	17.2	74.1	15.0	4.3	10.2
$HORRAT_R$	3.3	1.2	2.0	0.9	1.7	1.5	2.0	1.1

N = number of laboratories; S_r = repeatability standard deviation; S_R = reproducibility standard deviation.
RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation.
r = repeatability limit; R = reproducibility limit.

Table 5. Overview individual results - concentrations, corrected for recovery; expressed as mg HCN/kg.

Lab code	Chicken feed		Pig feed		Horse feed		Tapioca		Linseed		Almonds	
1	11.0	10.6	58.8	55.0	***49.4***	***50.7***	108.3	100.6	***142.9***	***142.3***	63.8	55.7
3	10.5	10.8	46.9	52.8	69.6	71.4	126.6	127.6	347.8	376.7	61.3	57.3
4	13.1	11.5	53.9	53.7	96.6	100.0	131.9	130.0	294.4	291.9	56.6	53.7
5	19.1	19.5	-	-	-	-	-	-	-	-	-	-
7	12.6	12.9	59.2	57.0	94.3	94.6	124.5	120.5	319.9	330.4	58.7	59.8
8	14.9	10.1	54.4	56.0	86.2	85.4	122.5	120.9	320.4	348.1	55.8	56.6
9	14.8	13.6	63.8	64.1	94.9	97.5	119.6	129.7	345.9	364.6	68.4	65.9
10	8.4	7.5	47.5	54.0	***52.0***	***55.0***	98.9	99.2	287.3	274.1	***42.3***	***38.4***
11	14.7	13.6	53.9	53.9	82.1	86.9	115.1	114.8	301.1	303.1	55.6	56.4
12	11.0	14.8	**61.9**	**16.4**	94.7	84.1	118.8	122.5	**213.2**	**303.8**	65.7	51.2
14	15.2	12.4	56.2	54.7	92.1	89.2	115.1	121.3	297.6	293.4	53.7	51.1
15	14.2	13.0	53.0	50.8	85.8	82.6	113.8	113.5	328.0	285.2	68.2	57.0
17	10.4	8.6	50.7	52.3	90.1	93.5	79.4[b]	-	340.8	336.2	64.3	62.2
18	10.8	8.7	61.5	51.8	91.4	79.4	134.2	134.0	256.3	209.8	69.3	58.3
19	13.5	14.0	57.6	58.7	91.7	92.1	122.0	119.6	326.3	336.3	59.2	60.4

*Outliers identified by Cochran (**bold**) and Grubbs (**italics, bold**).*
Outliers identified by operator: [b] single values.

Table 6. Results statistical analysis, corrected for recovery.

	Chicken feed	Pig feed	Horse feed	Tapioca	Linseed	Almonds
Mean (mg/kg)	12.5	55.1	88.6	119.4	313.2	59.5
N	15	14	14	13	14	14
Outliers	0	1	2	0	2	1
S_r (mg/kg)	1.4	2.8	3.7	3.1	16.3	4.7
S_R (mg/kg)	2.9	4.3	7.8	10.0	37.6	5.2
RSD_r (%)	11.4	5.1	4.2	2.6	5.2	7.9
RSD_R (%)	22.8	7.8	8.8	8.4	12.0	8.8
r (mg/kg)	4.0	7.8	10.4	8.6	45.6	13.1
R (mg/kg)	8.0	12.1	21.8	28.0	105.3	14.6
$HORRAT_R$	2.1	0.9	1.1	1.1	1.8	1.0

N = number of laboratories; S_r = repeatability standard deviation; S_R = reproducibility standard deviation.
RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation.
r = repeatability limit; R = reproducibility limit

Table 7. Overview individual results (in mg HCN/kg) lab 2.

Lab code	Blank feed		Chicken Feed		Pig Feed		Horse Feed		Tapioca		Linseed		Almonds		Blank feed spike 10 mg/kg		Blank feed spike 50 mg/kg	
2	<LOQ	<LOQ	11.5	11.3	55.7	53.1	87.3	86.0	108.2	105.1	298.1	271.8	54.5	50.5	8.8	8.2	46.9	43.1
2*			13.5	13.3	61.9	59.0	97.0	95.6	120.2	116.8	331.2	302.0	60.6	56.1				

** Lab results, corrected for recovery.*

3.7 Comments of the participants to the study

Participants have been asked to complete a questionnaire in order to comment on the method description, the execution of the method and organisation of the study.

In general to the questions the following remarks/comments have been made:

- Did you follow the procedure as described?

16 participating laboratories indicated that the provided description of the procedure has been followed or very minor modifications were made. One laboratory (lab 2) did not follow the procedure as described. The sample (at 8.3.1) was weighted directly into the steam-distillation bottles, instead of weighing in a screw cap bottle. This lab reported that they did not have access to screw cap bottles. The advantage, according to the lab, was a reduction of one preparation step: the transfer with cold water, after incubation and enzymatic breakdown, into a steam distillation bottom flask. Because the lab did not follow the method description, the results were excluded from statistical analysis.

- Was the method description adequate?

The description of the sample preparation procedure and analysis were adequately described. All of the laboratories could execute the procedure.

- Was there a need to adjust the procedure from that stated in the method description?

It was advised to use a 1 mol/l NaOH solution to adjust the pH of the EDTA solution (at 3.18). The concentration range of the standards (3.20) should be extended to a concentration level up to 1 µg/ml.

- Did you encounter any problems during execution of the method?

One lab reported that in some cases the stability of the derivative in the final sample extracts proved to be unsatisfactory over a longer period (24 hours) at room temperature. A note has been made in the final method for optional use of a cooled HPLC sampling tray.

- What type of steam distillation is used?

Different types of steam distillation equipment was used: conventional home made equipment, different types of Büchi distillation equipment, Kjeltec distillation equipment or Gerhard distillation equipment. All of these proved to be suitable for its purpose.

- What type of HPLC - (i) column and - (ii) equipment was used for the trial?

All the participants utilized normal HPLC equipment and HPLC columns packed with reversed phase C18 material.

- What criticisms and/or suggestions could you make concerning the method and/or its performance?

Suggestions for clarification of the calculation formula to calculate the concentration HCN in the sample were made.

- Did you have any comments to the organization (and/or execution) of the collaborative study?

No critical comments.

4 Conclusions

An HPLC method coupled to fluorescence detection for the determination of bound and free cyanide in various feeds and feed materials, has been validated via a collaborative study.

The relative standard deviation found for repeatability (RSD_r) ranged from 1.4 - 10.4% and the corresponding relative standard deviation of reproducibility (RSD_R) ranged from 7.2 - 38.8%. In general, the $HORRAT_R$ values were equal to or below the critical value of 2. One out of eight positive samples the $HORRAT_R$ value exceeded 2, the highest value being 3.3 for a chicken feed with a mean content of 7.7 mg/kg.

When correction for recovery rate was applied the relative standard deviation for repeatability (RSD_r) ranged from 2.6 - 11.4% and the corresponding relative standard deviation of reproducibility (RSD_R) ranged from 7.8 - 22.8%. Only for one out of six positive samples the $HORRAT_R$ value slightly exceeded the value of 2, viz. 2.1 for a chicken feed with a mean content of 12.5 mg/kg.

Recovery correction leads to improved performance characteristics of the method with regards to $HORRAT_R$ values and number of outliers. While recovery correction has to be applied because of EC-legislation, the recovery corrected figures for the performance characteristics should be included in the draft CEN-method.

The method proved to be applicable to analyse cyanide in various feeds and feed materials, viz. chicken feed, pig feed, horse feed, tapioca, linseed and almonds. The method is applicable in the concentration range of 2 - 400 mg/kg. The limit of determination of the method is 2 mg HCN/kg feed.

The proposed method is able to detect hydrocyanic acid at the limits according to European legislation viz. 10 mg HCN/kg for chicken feed and higher contents for other feeds and feed materials. The method description, revised following the comments of the participants, is included in Annex I.

Acknowledgements

The authors would like to thank the participating laboratories:

- AGES - Agency for Food and Health Safety (Vienna, Austria),
- Bavarian Health and Food Safety Authority (Oberschleissheim, Germany),
- Chemical and Veterinary control laboratory Münster (Münster, Germany),
- CVUA Sigmaringen (Sigmaringen, Germany),
- Laboratorio Arbitral Agroalimentario (Madrid, Spain),
- LAREAL (Saint Nolff, France),
- LTZ Augustenberg (Karlsruhe, Germany),
- LUFA Speyer (Speyer, Germany),
- Minton Treharne & Davies Ltd (Cardiff, United Kingdom),
- Minton Treharne & Davies Ltd (Llanelli, United Kingdom),
- National Reference laboratory (Budapest, Hungary),
- Oleotest (Antwerpen, Belgium).
- SCL-Laboratoire de Rennes (Rennes, France),
- The Danish Plant Directorate (Lyngby, Denmark),
- TUM, Bioanalytik (Freising, Germany),
- West Yorkshire Analytical Services (Leeds, United Kingdom),
- Worchester Scientific Services (Worchesterhire, United Kingdom).

Dr. Gerhart Thielert, CVUA Sigmaringen, Germany, dr. Age Jongbloed and Wim Traag, Wageningen UR, Wageningen, the Netherlands are thanked for supplying the feed materials for the collaborative studies.

Dr. Jörg Stroka (Joint Research Centre (JRC), IRMM, Geel, Belgium) is thanked for supplying the statistical evaluation programme. Dr. Jörg Stroka and Dr. Andreas Breidbach (JRC-IRMM) are thanked for their help in the statistical evaluation.

Dipl. Ing. Jürgen Danier (Bioanalytik Weihenstephan, Technische Universität München, Germany) is thanked for his help in the statistical evaluation

Ir. Jac Thissen, Wageningen UR, Wageningen, The Netherlands, is thanked for performing the Mandel graphical consistency technique and for further help in the statistical evaluation.

Alex Koot and Jan Kiers, RIKILT, are thanked for performing the homogeneity studies of the feed materials.

This project has been supported by the European Commission under the Mandate M 382 to CEN/TC 327.

References

1) Thompson, M. and R. Wood, 1993. The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories, J. AOAC INt., 76(4), 926-940.
2) ISO 13528:2005, Statistical methods for use in proficiency testing by interlaboratory comparisons.
3) Fearn, T. and M. Thompson, 2001. A New Test for 'Sufficient homogeneity', Analyst, 126, 1414-1417.
4) Thompson, M., 2000. Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations in relation to fitness for purpose criteria in proficiency testing, Analyst, 125, 385-386.
5) Horwitz, W., 1995. Protocol for the design, conduct and interpretation of method-performance studies. Pure & Appl. Chem., Vol. 67, No. 2, pp. 331-343.
6) Horwitz, W., L.R. Kamps and K.W. Boyer, 1980. Quality assurance in the analysis of foods and trace constituents. Journal of the Association of Official Analytical Chemistry 63: 1344-1354.
7) Horwitz, W. and R. Albert, 2006. The Horwitz ratio (HORRAT): A useful index of method performance with respect to precision. Journal of AOAC International 89: 1095-1109.
8) Commission of the European Communities, Commission Regulation (EC), 2009. No. 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed; Official Journal of the European Union L 54, 26.2.2009, p. 1-130.
9) ISO 5725-2, 1994. 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) Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

Annex I
Animal feeding stuffs - Determination of Hydrocyanic acid by HPLC

Draft of the method, including revisions following the comments of the participants of the collaborative study. Date: June 2010

NOTE: This protocol is not an official CEN-standard. The final CEN-standard, including the comments from the Enquiry stage, will be available in 2012.

1 Scope

This European Standard is applicable to the quantitative analysis of bound and free cyanide in animal feed and feed materials of plant origin.

The method is validated from 10 - 350 mg HCN/kg. When the method is used outside this range it should be validated. A limit of quantification of 2 mg HCN/kg should normally be achievable.

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, *Water for analytical laboratory use — Specification and test methods*

ISO 6498, *Animal feeding stuffs — Preparation of test samples*

3 Principle

Cyanide occurs in feed as cyanoglycosides. Cyanoglycosides are degraded by enzymes and cyanide is formed.

Cyanoglycosides are extracted from feed with an acid solution. After incubation with acid, the pH is adjusted to neutral and ß-glucosidase is added for enzymatic breakdown of cyanoglycosides at 38°C. Cyanide is extracted from the sample by steam distillation and collected in a potassium hydroxide solution. Subsequently, cyanide is derivatized with taurine and NDA (2,3 napthylene dicarboxy aldehyde) to form a fluorescent complex. The cyanide complex is analysed by HPLC with fluorescence detection.

4 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

WARNING — Use all solvents and solutions in a fume hood. Wear safety glasses, protective clothing, and avoid skin contact.

4.1 **β-glucosidase** from almonds, EC 3.2.1.21, minimum 2 units/mg (e.g. Sigma G-0395)

4.2 **Potassium cyanide**, KCN

4.3 **Amygdalin**, e.g. Sigma A-6005

4.4 **Potassium dihydrogen phosphate**, KH_2PO_4

4.5 **di-Potassium hydrogen phosphate**, K_2HPO_4

4.6 **Sodium hydroxide**, NaOH

4.7 **Methanol**, CH_3OH, HPLC grade

4.8 **Sodium acetate trihydrate**, $CH_3COONa*3H_2O$

4.9 **Acetic acid**, CH_3COOH

4.10 **Orthophosphoric acid**, H_3PO_4, 85% (~14,65 M)

4.11 **Sodium meta borate tetrahydrate**, $BNaO_2$ *4 H_2O

4.12 **NDA**, 2,3 Naphthalene dicarboxy aldehyde, $C_{12}H_8O_2$

4.13 **Taurine**, $C_2H_7NO_3S$

4.14 **EDTA**, Titriplex III - $C_{10}H_{14}N_2Na_2O_8*2H_2O$, (e.g. Merck art. No. 1.08418.0250)

4.15 **Orthophosphoric acid solutions**, concentrations c(H_3PO_4) = 0,1 mol/l and 0,02 mol/l:

- 0,1 mol/l: Add 6,9 ml H_3PO_4 85% (4.10) to a volumetric flask of 1000 ml containing 500 ml water, fill to mark with water and mix;
- 0,02 mol/l: Transfer 200 ml 0,1 mol/l H_3PO_4 solution to a volumetric flask of 1000 ml, fill to mark with water and mix.

Prepare these solutions for every series.

4.16 **Sodium hydroxide solutions**, concentrations c(NaOH) = 1,0 mol/l, 0,1 mol/l and 0,01 mol/l:

- 1,0 mol/l: Add 4,0 g Sodium hydroxide (4.6) to a volumetric flask of 100 ml containing 50 ml water, dissolve and fill to mark with water and mix.
- 0,1 mol/l: Transfer 100 ml 1,0 mol/l Sodium hydroxide solution to a volumetric flask of 1000 ml, fill to mark with water and mix.
- 0,01 mol/l: Transfer 100 ml 0,1 mol/l Sodium hydroxide solution to a volumetric flask of 1000 ml, fill to mark with water and mix.

These solutions are stable for three months.

4.17 **EDTA solution**, concentration = 0,20 mol/l

Weigh 37,2 g EDTA (4.14) in a 500 ml beaker, add 300 ml water and adjust the pH to 7,0-8,0 with a 1,0 mol/l and 0,1 mol/l Sodium hydroxide solution (4.16) (use a pH meter (5.12)) until dissolved. Transfer to a volumetric flask of 500 ml; fill to mark with water and mix. This solution is stable for one month.

4.18 Stock solution for standards

Weigh, to the nearest 0,1 mg, 100 mg KCN (4.2) in a 100 ml volumetric flask. Add 50 ml 0,01 mol/l Sodium hydroxide solution (4.16) and dissolve the KCN, fill to mark with 0,01 mol/l Sodium hydroxide (4.16) and mix. This is the stock solution of 400 µg CN^-/ml.

The solution is stable for 3 months when stored at 6°C ± 2°C.

4.19 Standards

Add, using a mechanic pipette (5.4), 1,00 ml stock solution for standards (4.18) in a 100 ml volumetric flask and fill to the mark with 0,01 mol/l Sodium hydroxide solution (4.16) and mix; this is the work solution of 4 µg CN^-/ml. Prepare standard solutions by following schedule in 100 ml volumetric flasks and using a mechanic pipette (5.4):

Table I.1. Calibration standard solutions.

	Amount work solution	Concentration (µg CN^-/ml)
Standard 1	25,00 ml	1,00
Standard 2	10,00 ml	0,40
Standard 3	5,00 ml	0,20
Standard 4	2,50 ml	0,10
Standard 5	1,25 ml	0,05
Standard 6	0,00 ml	0,00

Use 0,01 mol/l Sodium hydroxide solution (4.16) to fill the volumetric flasks to the mark.

For every series of analysis, fresh standards are prepared.

4.20 β-glucosidase solution

200 IU β-glucosidase (4.1) per ml water.

NOTE: The amount of ß-glucosidase weighed depends on the activity of the enzyme, given by the manufacturer of the enzyme. E.g. when the activity is 2 IU/mg enzyme, 100 mg enzyme/ml water is weighed. A new batch of β-glucosidase can be tested by analysing a sample with a known amount of cyanide e.g. a reference sample. If the enzyme is active enough, the expected amount of cyanide should be detected.

For every series of analysis, a fresh solution is prepared

4.21 **Amygdalin spike solution**, concentration c(amygdalin) 0,019 mol/l

Dissolve 85,0 mg amygdalin (4.3) in 10 ml water.

For every series of analysis, a fresh solution is prepared.

4.22 Sodium acetate solution, concentration c($CH_3COONa*3H_2O$) 0,75 mol/l

Dissolve 100 g sodium acetate trihydrate (4.8) in 800 ml water. Adjust pH to 7,9 with diluted (20x) acetic acid (4.9). Use a pH meter to control (5.12). Transfer to a volumetric flask of 1000 ml. Fill to the mark with water and mix.

The solution remains stable for 3 months when stored at room temperature.

4.23 Phosphate buffer for HPLC mobile phase, concentration c(PO_4) 0,05 mol/l

Dissolve 3,40 g Potassium dihydrogen phosphate (4.4) and 4,35 g di-Potassium hydrogen phosphate (4.5) in 100 ml water in a beaker. Transfer to a volumetric flask of 1000 ml, fill to mark with water and mix.

The solution remains stable for 3 months when stored at room temperature.

4.24 Phosphate buffer for NDA/taurine solution, concentration c(PO_4) 0,1 mol/l, concentration c($BNaO_2$) 0,025 mol/l

Dissolve 3,40 g Potassium dihydrogen phosphate (4.4), 4,35 g di-Potassium hydrogen phosphate (4.5) and 3,45 g Sodium meta borate tetrahydrate (4.11) in 100 ml water. Transfer to a volumetric flask of 500 ml. Fill to the mark with water and mix.

This solution remains stable for 3 months when stored at 6°C ± 2°C.

4.25 NDA solution; concentration c(NDA) 0,002 mol/l

Weigh 36,8 mg NDA (4.12) in a 100 ml volumetric flask. Add 40 ml methanol (4.7) and dissolve the NDA.

Fill to the mark with phosphate buffer for NDA/taurine solution (4.24) and mix.

This solution remains stable 3 months when stored at 6°C ± 2°C.

4.26 Taurine solution, concentration c(taurine) 0,05 mol/l

Weigh 0,626 g taurine (4.13) in a 100 ml volumetric flask. Add 40 ml Phosphate buffer for NDA/taurine solution (4.24) and dissolve taurine. Fill to the mark with phosphate buffer for NDA/taurine solution (4.24) and mix. This solution remains stable for 3 months when stored at 6°C ± 2°C.

4.27 HPLC mobile phase

Weigh 675 g methanol (4.7) in a flask of 2000 ml and add 1000 ml Phosphate buffer for HPLC mobile phase (4.23), mix and cool to room temperature. Filter the mobile phase using a filtrate system (5.6).

The solution remains stable for 3 months when stored at room temperature.

4.28 Liquid nitrogen

5 Apparatus

5.1 **Common laboratory glassware**, such as graduated cylinders, volumetric flasks, volumetric pipettes and screw cap glass bottles.

5.2 **Volume pipettes**, 5 ml and 10 ml

5.3 **Two neck round bottom flask of 1000 ml** or a sample tube of 500 ml

5.4 **Mechanic pipette**, 100-1000 µL

5.5 **Repeat pipette**

5.6 **Filtration system for HPLC mobile phase** with a membrane filter 0,45 µm

5.7 **Steam distillation unit**, conventional e.g. Landgraaf no AS136721111 or integrated e.g. Buchi K-350, or equivalent unit

5.8 **HPLC system**:

- Analytical pump;
- Column oven;
- Autosampler with a 10 µl sample loop; Optional: with cooled tray;
- HPLC Fluorescence detector;
- Analytical reversed phase HPLC column: C18 RP-column with column dimensions preferably 125 mm L x 4 mm I.D., stationary phase with particle size 5 µm;
- Precolumn: C18 RP-column with column dimensions preferably 7,5 mm L x 4 mm I.D., stationary phase with particle size 5 µm.

5.9 **Analytical balance**

5.10 **Balance**

5.11 **Mill**, capacity to grind to 1 mm

5.12 **pH meter**

6 Sampling

It is important that the laboratory receives a sample that is truly representative and has not been damaged or changed during transport and storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 6497 (1). It is recommended that samples be stored frozen to prevent changes in cyanide levels due to endogenous enzyme activity.

7 Preparation of test sample

Prepare the test sample in accordance with ISO 6498.

Grind the entire laboratory sample so it passes completely through a sieve with 1 mm apertures using liquid nitrogen (4.28). Mix thoroughly.

Optional: When samples with high oil content are milled at room temperature, an emulsion will be formed when adding phosphoric acid. The emulsion can have a negative effect on the reproducibility. To prevent this, liquid nitrogen (4.28) is used with grinding.

8 Quality control sample

It is recommended to analyse a reference sample with a known cyanide content in every analysis series, e.g. linseed. Preparation of this sample is the same as for the test samples.

9 Procedure

9.1 Precautions

Cyanide is very toxic. Protecting gloves should be used during the preparation of the samples and standards. Waste should be disposed separately from normal waste.

In case of methanol, one should use a fume cupboard. The distillation apparatus could be placed in a fume cupboard.

NOTE: Hydrocyanic acid is volatile above 26°C. Avoid loss of hydrocyanic acid during sample handling; in particular during manual operations at 9.3.3 (opening and closing of bottle) and 9.3.4 (transfer of sample solution).

9.2 General

Analyse in each series the following samples:

- Blank sample
- Blank animal feed spiked with 100 µl amygdalin spike solution (4.21) to 5 grams feed: corresponds to 10 mg HCN/kg feed.
- Blank animal feed spiked with 500 µl amygdalin spike solution (4.21) to 5 grams feed: corresponds to 50 mg HCN/kg feed.

9.3 Sample handling

NOTE: Hydrocyanic acid is volatile, it is important to carry out the laboratory experiments at a temperature below 26°C.

Follow for each test portion the following instructions:

9.3.1 Weighing

Weigh, to the nearest 0,01 g, 5,00 ± 0,3 g feed and blank feed in a 100 or 150 ml (250 ml) screw cap glass bottle. Add 80 ml of a cold solution (<10°C) of 0,02 mol/l Orthophosphoric acid (4.15) to the sample, close the bottle, and mix very well on a stirring device.

NOTE: For spikes of 10 mg and 50 mg CN/kg feed, pipet 100 µl and 500 µl amygdalin spike solution (4.21) to blank feed.

9.3.2 Acid incubation

Incubate for 1 hour at low temperature (<26°C), under continuous stirring. Add slowly, under continuous stirring a cold solution (<10°C) of 15 ml 0,20 mol/l EDTA solution (4.17), close the screw cap glass bottle - mix for 10 minutes. Then adjust the pH between 5,9 and 6,0 using 0,1 mol/l Sodium hydroxide (4.16) and a pH meter (5.12) to control. (It may take some time to adjust the pH because of the buffering capacity of the feed.)

9.3.3 Enzymatic breakdown

Add 0,5 ml ß-glucosidase solution (4.20) and immediately close the screw cap glass bottle. Place the screw cap glass bottle in an oven of 38°C and incubate overnight under continuous shaking. After incubation, the screw cap glass bottle is cooled on ice to a temperature lower than 10°C.

9.3.4 Steam distillation

Transfer the sample to a neck round bottom flask (5.3). Use no more than 50 ml cold water (<10°C) for the transfer of the sample. Add 10 ml of a cold sodium acetate solution (4.22) (<10°C) to the sample and connect the neck round bottom flask (5.3) to the steam distillation unit (5.7). Collect distillate1) in a 250 ml or 500 ml volumetric flask that contains 25 ml or 50 ml of a cold solution (<10°C) of 0,1 mol/l Sodium hydroxide (4.16) and 25 ml or 80 ml cold water (<10°C). Fill the flask up to the mark with cold water (<10°C). The final concentration Sodium hydroxide in the volumetric flask should not exceed 0,01 mol/l. If necessary dilute the sample with 0,01 mol/l Sodium hydroxide (4.16).

NOTE: Rinse/clean the capillary of the steam distillation equipment after each distillation run to avoid cross contamination.

9.4 HPLC analysis

9.4.1 Derivatization

Pipette 0,5 ml sample (9.3) into a HPLC vial. Subsequently, add 100 µl taurine solution (4.26) and 100 µl NDA solution (4.25) to the sample. Close the vial, mix and leave for 30 minutes at room temperature. Place the vial in the tray of the autosampler. Protect the vials for UV/visible light.

---

1) Approximately 200 ml - 300 ml when using conventional steam distillation equipment or less if using modern equipment.

The derivatization procedure is also applied to the standards (4.19).

Optional: When a cooled HPLC autosampler tray has been used, the derivates are stable for at least 24 hours.

9.4.2 HPLC conditions

Prior to each series of analysis, the column is first flushed with water and methanol (4.7) and then with mobile phase until pressure and base line are stabilized.

- Pump:
  - flow : 1,0 ml/min;
  - column temp : 30°C;
  - time of analysis: 15 minutes, cyanide peak should occur between 4 min. and 10 min.
- Auto sampler:
  - injection volume: 10 µl.
- Fluorescence detector:
  - excitation : 418 nm;
  - emission : 460 nm;
  - gain : 10;
  - response : slow.

9.4.3 Analysis

Prior to analysis: inject 10 µl of Standard 1 (1,00 µg CN^-/ml) and Standard 5 (0,05 µg CN^-/ml) (4.19). A single peak should be observed and the peak area ratio of the two standards should be correct.

HPLC analysis: Standards followed by spiked samples, samples and standards.

10 Calculation of results

Determine the area of the CN-peak (if available use data acquisition software). Use the cyanide standards for calculation of a 6-points calibration line (peak area vs. concentration) using linear regression.

Use the resulting function (y=ax+b) to calculate the concentration of CN^- in the measured test solution (where a is the value of the slope of the linear function and b is the value where the calibration function intercepts the y-axis of the co-ordinate system).

NOTE: Calculate the correlation coefficient R^2. Criterium for correlation coefficient: >0,995.

Convert the concentration of CN^- into a concentration expressed as HCN. Finally, if the recovery falls outside the range of 90% - 110%, the results must be corrected for recovery.

Correction of recovery can be performed in several ways:

- By means of spiking of a blank sample. Preferably a blank sample similar in type to that of the analysed sample should be selected. The spiked level should be similar to the concentration of the analysed sample;
- By means of the standard addition procedure. The concentration added to the analysed sample should be similar to the concentration found in the analysed sample.

10.1 Formula

Calculation of the hydrocyanic acid mass fraction of the sample using the following equations:
Calculation of the calibration curve (function) obtained by linear regression:

$$A\ (mg/ml) = a \times Signal\,(units) + b \quad (1)$$

Calculate the content of HCN in the test material according to:

$$E\,(mg\ HCN/kg) = (A \times B \times C \times 1{,}04)/D \quad (2)$$

where:

A *is the concentration of CN- in the injected solution calculated from linear regression;*

B *is the ml final volume of the volumetric flask at 9.3.4 (250 or 500);*

C *is the dilution factor (if applicable);*

D *is the test portion mass (g);*

E *is the HCN mass fraction of the sample (mg/kg).*

11 Precision

11.1 Collaborative study

A collaborative study was carried out in 2009. Details of the tests on precision of the method are summarised in Annex A. The values derived from these tests may not be applicable to concentration ranges and matrices others than those given.

11.2 Repeatability

The absolute difference between two independent single test results, obtained using the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval time, will in no more than 5% of the cases be greater than the repeatability limit r given in Table I.2 - precision data.

11.3 Reproducibility

The absolute difference between two single test results obtained using the same method on identical test material in the same laboratory with different operators using different equipment, will in no more than 5% of the cases be greater than the reproducibility limit R given in Table I.2 - precision data.

Table I.2. Precision data (results statistical analysis corrected for recovery).

	Chicken feed	Pig feed	Horse feed	Tapioca	Linseed	Almonds
Mean (mg/kg)	12,5	55,1	88,6	119,4	313,2	59,5
r (mg/kg)	4,0	7,8	10,4	8,6	45,6	13,1
R (mg/kg)	8,0	12,1	21,8	28,0	105,3	14,6

12 Test report

The test report shall specify:

1. information necessary for complete identification of the sample;
2. the method with which sampling was carried out, if known;
3. the method used;
4. the test result obtained;
5. if the repeatability has been checked, the final quoted result obtained;
6. operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

Annex A
Results of the collaborative study (informative)

A collaborative study was carried out in 2009 with 17 participating laboratories and 6 different animal feedingstuffs, including complete feeds for chicken, pigs and horses, tapioca, linseed and almonds. The samples were homogenised centrally and distributed to the participants. Statistical evaluation was performed according to ISO 5725-2 (5).

The following data were obtained:

Table A.1. Statistical results of collaborative study (corrected for recovery).

	Chicken feed	Pig feed	Horse feed	Tapioca	Linseed	Almonds
Number of laboratories	**17**	**16**	**16**	**16**	**16**	**16**
Number of non-compliant laboratories	**2**	**2**	**2**	**3**	**2**	**2**
Number of outliers	**0**	**1**	**2**	**0**	**2**	**1**
Number of accepted results	**15**	**13**	**12**	**13**	**12**	**13**
Mean (mg/kg)	**12,5**	**55,1**	**88,6**	**119,4**	**313,2**	**59,5**
S_r (mg/kg)	**1,4**	**2,8**	**3,7**	**3,1**	**16,3**	**4,7**
S_R (mg/kg)	**2,9**	**4,3**	**7,8**	**10,0**	**37,6**	**5,2**
RSD_r (%)	**11,4**	**5,1**	**4,2**	**2,6**	**5,2**	**7,9**
RSD_R (%)	**22,8**	**7,8**	**8,8**	**8,4**	**12,0**	**8,8**
r (mg/kg)	**4,0**	**7,8**	**10,4**	**8,6**	**45,6**	**13,1**
R (mg/kg)	**8,0**	**12,1**	**21,8**	**28,0**	**105,3**	**14,6**
$HORRAT_R$	**2,1**	**0,9**	**1,1**	**1,1**	**1,8**	**1,0**

S_r = repeatability standard deviation; S_R = reproducibility standard deviation;
RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation;
r = repeatability limit; R = reproducibility limit; $HORRAT_R$ = HORRAT value for reproducibility.

Annex B Results of the collaborative study without recovery correction (informative)

The following table is shown purely for informative purposes. It lists the statistical results of the same collaborative study as reported in Annex A but without recovery correction. The results show that, in order to reach optimal results, recovery correction is necessary.

Table B.1. Statistical results of collaborative study (not corrected for recovery).

	Chicken feed	Pig feed	Horse feed	Tapioca	Linseed	Almonds	Feed 10 ppm	Feed 50 ppm
Number of laboratories	**17**	**17**	**17**	**17**	**17**	**17**	**17**	**16**
Number of non-compliant laboratories	**1**	**1**	**1**	**2**	**1**	**1**	**2**	**2**
Number of outliers	**1**	**2**	**1**	**2**	**3**	**1**	**1**	**1**
Number of accepted results	**15**	**14**	**15**	**13**	**13**	**15**	**14**	**13**
Mean (mg/kg)	**7,7**	**38,5**	**58,4**	**84,7**	**227,4**	**39,9**	**6,3**	**35,0**
S_r (mg/kg)	**0,8**	**1,8**	**2,3**	**1,2**	**14,6**	**2,9**	**0,5**	**1,6**
S_R (mg/kg)	**3,0**	**4,2**	**9,9**	**6,1**	**26,5**	**5,4**	**1,5**	**3,7**
RSD_r (%)	**10,4**	**4,7**	**3,9**	**1,4**	**6,6**	**7,2**	**7,6**	**4,6**
RSD_R (%)	**38,8**	**10,9**	**17,0**	**7,2**	**11,9**	**13,4**	**24,2**	**10,5**
r (mg/kg)	**2,3**	**5,1**	**6,4**	**3,4**	**40,9**	**8,0**	**1,3**	**4,5**
R (mg/kg)	**8,4**	**11,8**	**27,7**	**17,2**	**74,1**	**15,0**	**4,3**	**10,2**
$HORRAT_R$	**3,3**	**1,2**	**2,0**	**0,9**	**1,7**	**1,5**	**2,0**	**1,1**

S_r = repeatability standard deviation; S_R = reproducibility standard deviation;
RSD_r = repeatability relative standard deviation; RSD_R = reproducibility relative standard deviation;
r = repeatability limit; R = reproducibility limit; $HORRAT_R$ = HORRAT value for reproducibility.

Annex C
Alternative procedure for enzymatic breakdown (par. 9.3.3) and steam distillation (par. 9.3.4) (informative)

The following alternative procedure may yield satisfactory results but has not been fully validated through the collaborative study:

The sample is weighted (at 9.3.1 of the procedure) directly into steam-distillation bottles which might be of an automated system too instead of weighing in a screw cap glass bottle.

During the enzymatic breakdown (9.3.3) step the steam-distillation bottle is tightly closed with stoppers and additionally sealed with Parafilm. After enzymatic breakdown, the steam-distillation (9.3.4) can be done directly in this steam-distillation bottle.

NOTE: In the collaborative study one laboratory applied this alternative procedure. The results obtained were as follows:

Table C.1. Laboratory results (mg HCN/kg).

Chicken Feed		Pig Feed		Horse Feed		Tapioca		Linseed		Almonds		Blank feed spike 10 mg/kg		Blank feed spike 50 mg/kg	
11.5	11.3	55.7	53.1	87.3	86.0	108.2	105.1	298.1	271.8	54.5	50.5	8.8	8.2	46.9	43.1
13.5	*13.3*	*61.9*	*59.0*	*97.0*	*95.6*	*120.2*	*116.8*	*331.2*	*302.0*	*60.6*	*56.1*				

All samples are analysed in duplicate. The Italics results are corrected for recovery.

The recovery corrected results fitted well into the range of results obtained by other laboratories in the collaborative study (see Annex A).

Bibliography

1) ISO 6497, 2002. Animal feeding stuffs - Sampling.
2) S. Chinaka, N. Takayama, Y. Michigami and K. Ueda. Simultaneous determination of cyanide and thiocyanate in blood by ion chromatography with fluorescence and ultraviolet detection, J. of Chrom. B 1998, 713, pp. 353-359.
3) A. Sano, N. Takimoto and S. Takitani. High-performance liquid chromatographic determination of cyanide in human red blood cells by pre-column fluorescence derivatization, J. of Chrom. 1992, 582, pp. 131-135.
4) 12-7-71 Publicatieblad van de Europese Gemeenschappen Nr. L 155/15.
5) ISO 5725-2, 1994. 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.

RIKILT - Institute of Food Safety is part of the international knowledge organisation Wageningen UR (University & Research centre). RIKILT conducts independent research into the safety and quality of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

RIKILT advises national and international governments on establishing standards and methods of analysis. RIKILT is available 24 hours a day and seven days a week in cases of incidents and food crises.

The research institute in Wageningen is the National Reference Laboratory (NRL) for milk, genetically modified organisms, and nearly all chemical substances, and is also the European Union Reference Laboratory (EU-RL) for substances with hormonal effects.

RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

More information: www.rikilt.wur.nl