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Fertilizers and soil conditioners — Determination of biuret content of urea-based fertilizers — HPLC method

Engrais et amendements — Détermination de la teneur en biuret des engrais à base d'urée — Méthode HPLC

Reference number
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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information.

The committee responsible for this document is ISO/TC 134, *Fertilizers and soil conditioners*.

Introduction

Biuret, also known as 2-imidodicarbonic diamide ($NH_2CONHCONH_2$), is one of several by-products formed when molten urea is heated near or above its melting point (132 °C) during the manufacturing of urea.[1][2] The exact mechanism of biuret damage to different plants is still under investigation, but the harmful effects of high concentrations have been well documented, and many regulations/standards concerning the maximum allowed concentrations and/or the analytical methods have been published around the world.[1][3][4][5][6][7][8] Nowadays, there are at least three kinds of analytical methods available for the determination of biuret in fertilizers, including traditional spectrophotometric methods,[5][7] the atomic absorption spectrophotometric method,[8] and HPLC methods.[2][5][10][11] Recently, HPLC methods have shown superiority over other types of methods, owing to their ability to quantitatively determine biuret content by completely separating biuret from numerous urea-condensates. ISO/TC 134 is well aware of great efforts made by analysts/scientists around the world on separately seeking a uniform, quick and accurate method for the determination of biuret in fertilizers and attempted to unify the HPLC method to the greatest extent herein, based on the preliminary research by the China, US, and European experts.[2][5][10][11]

Fertilizers and soil conditioners — Determination of biuret content of urea-based fertilizers — HPLC method

1 Scope

This International Standard specifies the test procedure for determination of the biuret content in liquid and solid urea-based fertilizers based on the HPLC method.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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*

3 Principle

The biuret content in the fertilizer is extracted by aqueous acetonitrile mobile phase and separated from other contents by reversed liquid chromatography on an amino/aminopropyl column. The peak is detected by UV detector attached to the HPLC.

4 Reagents

WARNING — Acetonitrile is flammable and toxic. Refer to applicable Safety Data Sheet (SDS). The related operations shall be performed in the fume hood. This International Standard does not point out all possible safety problems and the user shall bear the responsibility to take proper safety and health measures and ensure the operations are compliant with the conditions stipulated by the related laws and regulations of the state.

Use only water conforming to grade 3 of ISO 3696.

4.1 Acetonitrile, HPLC grade.

4.2 Mobile phase, 150 ml water + 850 ml acetonitrile, filtered by 0,22 µm membrane as pre-treatment, and ultrasonic degas for 10 min before use.

NOTE An alternative method using acetonitrile-free mobile phase is listed in Annex B, with some limitations on the scope of application.

4.3 Biuret stock solutions (0,5 mg/ml = 500 ppm), weigh 0,500 0 g high purity Biuret, dissolved by mobile phase (4.2), and transferred into a 1 l volumetric flask, dilute to volume with mobile phase (4.2) and mix.

The biuret of at least 97 % purity and the biuret purity claimed on the label shall be based on the biuret content, not on the N-content. There are methods available for the purification procedure of biuret such as those described in AOAC 960.04A(c) or ISO 17322.

5 Apparatus

5.1 Ordinary laboratory apparatus.

5.2 Ultrasonic bath.

5.3 High-performance liquid chromatography instrument, with UV detector.

5.4 Microsyringe, 5 µl ~ 50 µl.

5.5 Syringe-driven filter, with organic filter membrane of 0,22 µm pores.

5.6 Injection loop, volume of 10 µl.

5.7 Sieve, with the aperture size of 0,50 mm.

6 Procedure

6.1 Preparation of test sample

For urea fertilizers, simply take 500 g of divided sample as the test portion; for compound fertilizers, take a reduced laboratory sample of 100 g, and grind until it passes through a sieve of aperture size 0,5mm. Mix thoroughly to homogenize the sample. Place in a clean dry bottle with lid.

6.2 Preparation of test solution

Duplicate replicate experiments shall be done for the final determination of result.

Weigh 0,1 g ~ 0,5 g test sample (accurately to 0,000 2 g, with biuret content of 1 mg ~ 2 mg ca.) into a 25 ml beaker. Add 10 ml mobile phase (4.2) and dissolve using an ultrasonic bath for 10 min. Transfer to a 25 ml volumetric flask and dilute to volume with mobile phase (4.2). Mix thoroughly and leave standing. Filter with a syringe filer to obtain the test solution.

6.3 Preparation of biuret working standard solutions

According to Table 1, pipette 0,00 ml, 0,50 ml, 1,00 ml, 3,00 ml, 5,00 ml and 10,00 ml biuret stock solution (4.3) into six separate 25 ml volumetric flasks. Dilute with respective volumes of mobile phase (4.2) and make up to the mark and mix thoroughly. Filter with 0,22 µm organic filter membrane.

Table 1 — Biuret working standard solutions

Volume of biuret stock solution ml	**Mass of biuret** mg
0,00[a]	0,00
0,50	0,25
1,00	0,50
3,00	1,50
5,00	2,50
10,00	5,00
[a] Blank solution.	

6.4 HPLC conditions

Recommended operating conditions of HPLC are listed in Table 2. Other HPLC conditions that can achieve the same separation effects may be used.

Table 2 — Recommended operating conditions of HLPC

Chromatographic column	Amino column or Aminopropyl column,[a] 4,6 mm × 250 mm, with 5 µm packing
Flow rate	1,0 ml/min ~ 1,3 ml/min
Inject volume	10 µl
Column temperature	35 °C
Detector wavelength	195 nm

[a] If the column is new or has not been in service for more than a week, condition the column for 4 h at room temperature with LC-grade isopropanol at a flow rate that will maintain at least 200 bars column back pressure. This is typically 1 ml/min. Wash the column again for 4 h with 100 % LC-grade acetonitrile at flow rate of 1 ml/min followed by washing with the mobile phase at flow rate of 1 ml/min until a stable base line is achieved.

NOTE 1 The best separation condition can be determined according to different equipment and situations.

NOTE 2 Depending on the type of urea-based fertilizers, other conditions may apply.

NOTE 3 Alternative method using acetonitrile-free mobile phase is listed in Annex B.

6.5 Preparation of standard curve

Ensure that the HPLC apparatus operating conditions are optimized. Successively inject 10 µl working standard solution (6.3) and determine the series of biuret working standard solution. Each working standard solution shall be determined two times. Draw the standard curve or get the linear regression equation by calculating the average peak areas of the biuret and the corresponding mass.

6.6 Determination of the biuret content in the test solution

Determine the test solution (6.2) with the same method of the working standard solution, measure the peak area, and calculate the biuret mass in each test solution according to the standard curve or linear regression equation. After completing the determination, first wash the system with mobile phase (4.2) for 30 min, then wash the system with absolute acetonitrile (4.1) for 30 min. Finally, turn off the apparatus according to the operating procedures.

6.7 Calculation and expression of the results

The mass fraction of biuret (%), w, is calculated as given in Formula (1):

$$w = \frac{m_1 \times 10^{-3}}{m} \times 100 \qquad (1)$$

where

m_1 is the mass of biuret, in mg, of the test solution, calculated according to the standard curve or linear regression equation corresponding to the peak areas;

m is the mass, in g, of the test portion.

The determination result is the arithmetic average of the parallel determination results.

6.8 Precision

6.8.1 Ring test

Details of ring test on the precision of the method are summarized in Annex A.

6.8.2 Repeatability, *r*

For all levels, the repeatability limit *r* is 0,022, with the unit of mass fraction (%).

6.8.3 Reproducibility, *R*

For all levels, the reproducibility limit *R* is 0,102, with the unit of mass fraction (%).

7 Test report

The test report shall contain at least the following information:

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

b) test method used with reference to this International Standard, i.e. ISO 18643:2016;

c) test results obtained;

d) date of sampling and sampling procedure (if known);

e) date when the analysis was finished;

f) whether the requirement of the repeatability limit has been fulfilled;

g) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents occurred when performing the method, which might have influenced the test results.

Annex A
(informative)

Report of international laboratories ring test

A.1 General

a) International laboratories ring tests based on ISO/CD 18643 were conducted between January 2014 and March 2014. Thirteen laboratories participated in the two parallel tests on seven test samples.

b) The test method described in ISO/CD 18643 was adopted here for the determination of biuret contents in the fertilizer samples.

c) Seven different types of fertilizer samples were used during the ring test, each with several mean levels. The test samples were sample A-NPK compound fertilizer, sample B-urea formaldehyde complex fertilizer, sample C-urea, sample D-NPK complex fertilizer, sample E-urea ammonium nitrate (UAN) solution, sample F-polymer sulfur coated urea (PSCU), and sample G-urea formaldehyde slow release liquid fertilizer (Trisert®[1)]). The biuret contents in all of the seven fertilizer samples lie in the range of 0,09 % ~ 1,01 % (mass fraction).

d) The precision of the test results was evaluated based on ISO 5725-2.

A.2 Statistical analysis of the test results of biuret contents

A.2.1 Original test results

Thirteen laboratories participated in the determination of biuret contents in fertilizer test samples. The results are listed in Table A.1, with the unit of mass fraction (%).

Table A.1 — Original test results of the determination of biuret contents

Results in mass fraction (%)

Laboratory *i*	Level *j*													
	A		B		C		D		E		F		G	
1	0,615	0,602	0,505	0,509	1,006	0,995	0,300	0,310	0,231	0,225	0,946	0,945	0,093	0,091
2	0,580	0,610	0,580	0,580	0,960	1,050	0,340	0,350	0,290	0,280	0,980	0,970	0,130	0,120
3	0,653	0,619	0,485	0,516	0,970	0,938	0,276	0,271	0,211	0,185	0,788	0,784	0,067	0,070
4	0,651	0,654	0,552	0,547	1,047	1,057	0,326	0,336	0,250	0,249	1,061	1,015	0,104	0,106
5	0,625	0,614	0,509	0,510	1,013	1,015	0,277	0,279	0,219	0,212	0,975	0,985	0,070	0,068
6	0,635	0,622	0,523	0,532	1,035	1,038	0,319	0,314	0,248	0,249	0,982	0,985	0,106	0,106
7	0,644	0,644	0,547	0,548	1,053	1,050	0,323	0,325	0,244	0,238	0,961	0,965	0,094	0,093
8	0,610	0,620	0,500	0,500	0,980	0,980	0,310	0,310	0,220	0,220	0,950	0,940	0,100	0,100
9	0,600	0,590	0,500	0,490	0,890	0,900	0,290	0,300	0,210	0,210	0,870	0,870	0,090	0,090
10	0,590	0,590	0,600	0,610	1,110	1,090	0,350	0,350	0,300	0,300	0,940	0,980	0,150	0,150
11	0,609	0,613	0,505	0,504	1,010	1,006	0,300	0,301	0,233	0,231	0,937	0,939	0,091	0,093
12	0,614	0,615	0,503	0,500	1,010	1,007	0,314	0,313	0,231	0,234	0,934	0,930	0,101	0,100
13	0,618	0,620	0,515	0,498	1,001	1,010	0,309	0,318	0,215	0,217	0,976	0,911	0,086	0,091

1) Trisert® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

A.2.2 Cell means by each laboratory

The cell means of analyses by each laboratory for the determination of biuret contents are listed in Table A.2, with the unit of mass fraction (%).

Table A.2 — Cell means of the determination of biuret contents

Results in mass fraction (%)

Laboratory *i*	Level *j*						
	A	B	C	D	E	F	G
1	0,609	0,507	1,001	0,305	0,228	0,946	0,092
2	0,595	0,580	1,005	0,345	0,285	0,975	0,125
3	0,636	0,501	0,954	0,274	0,198	0,786	0,069
4	0,653	0,550	1,052	0,331	0,250	1,038	0,105
5	0,619	0,510	1,014	0,278	0,215	0,980	0,069
6	0,629	0,528	1,037	0,317	0,249	0,984	0,106
7	0,644	0,548	1,051	0,324	0,241	0,963	0,093
8	0,615	0,500	0,980	0,310	0,220	0,945	0,100
9	0,595	0,495	0,895	0,295	0,210	0,870	0,090
10	0,590	0,605	1,100	0,350	0,300	0,960	0,150
11	0,611	0,505	1,008	0,301	0,232	0,938	0,092
12	0,615	0,502	1,009	0,314	0,233	0,932	0,101
13	0,619	0,507	1,006	0,314	0,216	0,944	0,089

A.2.3 Cell absolute differences of the analyses by each laboratory

The cell absolute differences of the analyses by each laboratory for the determination of biuret contents are listed in Table A.3, with the unit of mass fraction (%).

Table A.3 — Cell absolute differences of the determination of biuret contents

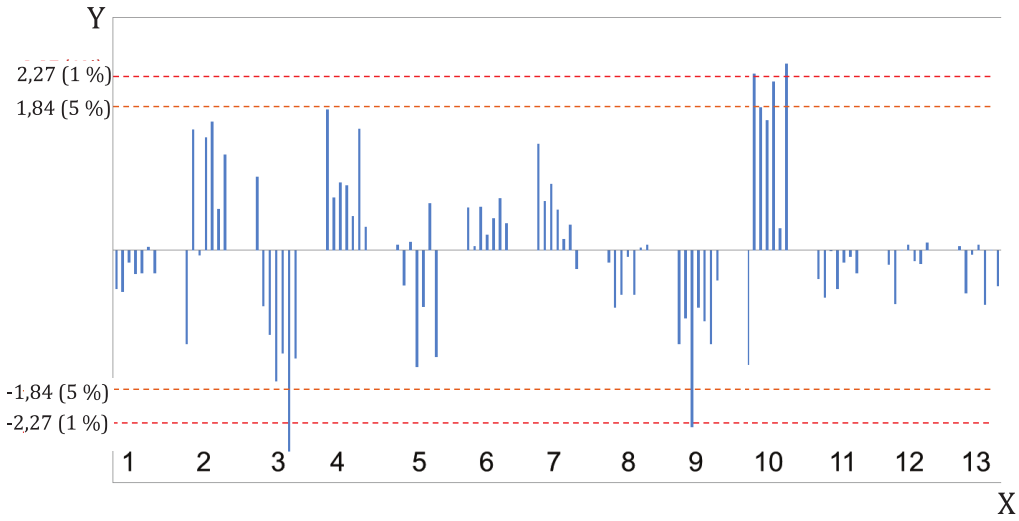
Results in mass fraction (%)

Laboratory *i*	Level *j*						
	A	B	C	D	E	F	G
1	0,013	0,004	0,011	0,010	0,006	0,001	0,002
2	0,030	0,000	0,090	0,010	0,010	0,010	0,010
3	0,034	0,031	0,032	0,005	0,026	0,004	0,003
4	0,003	0,005	0,010	0,010	0,001	0,046	0,002
5	0,012	0,001	0,002	0,002	0,007	0,009	0,001
6	0,013	0,009	0,003	0,005	0,001	0,003	0,000
7	0,000	0,001	0,003	0,002	0,005	0,004	0,001
8	0,010	0,000	0,000	0,000	0,000	0,010	0,000
9	0,010	0,010	0,010	0,010	0,000	0,000	0,000
10	0,000	0,010	0,020	0,000	0,000	0,040	0,000
11	0,004	0,001	0,004	0,001	0,002	0,002	0,002
12	0,001	0,003	0,003	0,001	0,003	0,004	0,001
13	0,002	0,017	0,009	0,009	0,002	0,065	0,005

A.2.4 Evaluation of the results for consistency and outliers

Graphical evaluation of the analytical results for consistency by Mandel's *h* and *k* statistics were studied.

The inter-laboratory consistency statistic h and the intra-laboratory consistency statistic k for each level of each laboratory were calculated. The h and k values for each cell for the respective laboratories were plotted to obtain the Mandel's h and k graphs.

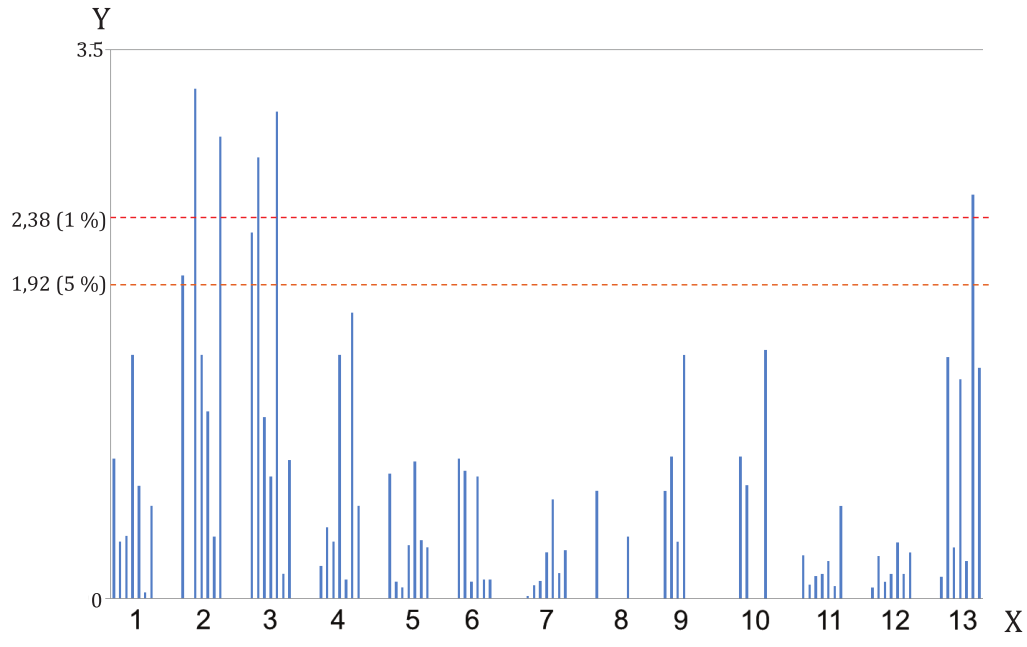


Key

X laboratory, *i*

Y Mandels's statistic, *h*

Figure A.1 — Mandel's inter-laboratory consistency statistic, *h*, grouped by laboratories



Key

X laboratory, *i*

Y Mandels's statistic, *k*

Figure A.2 — Mandel's intra-laboratory consistency statistic, *k*, grouped by laboratories

Horizontal dotted lines in Figure A.1 and Figure A.2 represent 1 % and 5 % critical values of Mandel's *h* and *k* statistics, respectively.

The Mandel's inter-laboratory consistency statistic *h* graph indicated that laboratory 10 had two stragglers on level C and level E, respectively; while laboratory 3 had an outlier on level F, laboratory 9 had an outlier on level C, and laboratory 10 had two outliers on level B and level G.

The Mandel's intra-laboratory consistency statistic *k* graph did exhibit rather large variability between replicate test results for laboratory 2 and 3 on level A; while very large variability between replicate test results for laboratory 2 on levels C and G, laboratory 3 on levels B and E, as well as laboratory 13 on level F.

Cochran' s test

Cochran's test is a test of the intra-laboratory variability and should be applied first, then any necessary action should be taken, and also with repeated tests, if necessary.

Application of Cochran's test led to the values of the test statistic C given in Table A.4.

Table A.4 — Values of Cochran's test statistic, C

Level *j*	A	B	C	D	E	F	G	Type of test
C	0,419	0,607	0,812	0,185	0,741	0,510	0,670	Cochran's test statistics
Stragglers (p = 13, n = 2) (5 %)	0,515	0,515	0,515	0,515	0,515	0,515	0,515	Cochran's critical values
Outliers (p = 13, n = 2) (1 %)	0,624	0,624	0,624	0,624	0,624	0,624	0,624	

If the test statistic is greater than its 5 % critical value and less than or equal to its 1 % critical value, the item tested is regarded as a straggler.

If the test statistic is greater than its 1 % critical value, the item tested is regarded as an outlier.

Cochran's test showed that the test statistic reached 0,818, calculated by the maximum cell absolute difference (0,090) from laboratory 2 on level C.

The Cochran's critical value at the 1 % significance level was 0,624, for p = 13 and n = 2; therefore, the test result from laboratory 2 on level C is an outlier, which should be discarded here.

Cochran's test showed that the test statistic reached 0,741, calculated by the maximum cell absolute difference (0,026) from laboratory 3 on level E.

The Cochran's critical value at the 1 % significance level was 0,624, for p = 13 and n = 2; therefore, the test result from laboratory 3 on level E is an outlier, which should be discarded here.

Cochran's test showed that the test statistic reached 0,670, calculated by the maximum cell absolute difference (0,010) from laboratory 2 on level G.

The Cochran's critical value at the 1 % significance level was 0,624, for p = 13 and n = 2; therefore, the test result from laboratory 2 on level G is an outlier, which should be discarded here.

Accordingly, level C and G were eliminated from the results for lab 2, and for lab 3, level E was eliminated from their results.

Cochran's tests (p = 12, n = 2) were repeated on the remaining tests values from the 12 laboratories on level C, E and G. The test statistic obtained this time were 0,547, 0,423 and 0,507, respectively. All of

these three values are less than the Cochran's critical value at the 1 % significance level (0,653, p = 12, n = 2). This confirmed that no outlier existed anymore.

Grubbs' test

The Grubbs' test is primarily a test of inter-laboratory variability. The test data used herein are those which have passed the Cochran's test.

Application of Grubbs' test to cell means led to the values of the test statistic G shown in Table A.5.

Table A.5 — Application of Grubbs' test to cell means

Level j;p	Single low	Single high	Double low	Double high	Type of test
A;13	1,456	1,845	0,652	0,475	Grubbs' test statistics
B;13	0,883	2,277	0,869	0,254	
C;12	2,191	1,758	0,595	0,367	
D;13	1,692	1,677	0,495	0,515	
E;12	1,071	2,161	0,792	0,206	
F;13	2,597	1,571	0,207	0,719	
G;12	1,334	2,587	0,618	0,287	
Stragglers (5 %)					Grubbs' critical values
p = 12	2,412	2,412	0,253 7	0,253 7	
p = 13	2,462	2,462	0,283 6	0,283 6	
Outliers (1 %)					
p = 12	2,636	2,636	0,173 8	0,173 8	
p = 13	2,699	2,699	0,201 6	0,201 6	

For the Grubbs' test for one outlying observation, outliers and stragglers give rise to values which are larger than its 1 % and 5 % critical values respectively.

For the Grubbs' test for two outlying observation, outliers and stragglers give rise to values which are smaller than its 1 % and 5 % critical values, respectively.

Application of Grubbs' test to the cell means confirmed that there was no outlier.

A.2.5 Calculation of the general mean and standard deviations

Calculation of the general mean, s_r, s_R of biuret contents in each sample listed in Table A.6, with the unit of mass fraction (%).

Table A.6 — Calculation results of the general mean, s_r, s_R of biuret contents

Results in mass fraction (%)

Sample/level	A	B	C	D	E	F	G
Number of laboratories	13	13	12	13	12	13	12
Outliers	0	0	1	0	1	0	1
General mean, m	0,62	0,53	1,01	0,31	0,24	0,94	0,10
Repeatability standard deviation, s_r	1,030E-2	7,806E-3	8,836E-3	4,563E-3	3,138E-3	1,786E-2	1,433E-3
Reproducibility standard deviation, s_R	2,029E-2	3,525E-2	5,223E-2	2,294E-2	2,794E-2	6,176E-2	3,573E-2

A.2.6 Dependence of precision on general mean (level), *m*

From Table A.6, it is clear that the standard deviations have no significant linear or logarithmic-linear relationship with the general mean (level), *m*.

For all levels, the mean value of the repeatability standard deviation, s_r, is 7,705E-3.

For all levels, the mean value of the reproducibility standard deviation, s_R, is 3,659E-2.

A.2.7 Final values of precision

The precision of the biuret contents measurements were discerned from Table A.6.

The final values of precision above were determined from a uniform-level experiment involving 13 laboratories, in which one test value from laboratory 2 on level C, one test value from laboratory 3 on level E and one test value from laboratory 2 on level G have been discarded as outliers.

For all levels, the mean value of the repeatability standard deviation, s_r, is 7,705E-3.

For all levels, the mean value of the reproducibility standard deviation, s_R, is 3,659E-2.

A.3 Conclusion

International laboratories ring tests on the determination of biuret contents in fertilizer test samples based on ISO/CD 18643 were carried out.

Thirteen laboratories around the world participated in the ring tests and contributed their data to the project.

Statistical evaluations on the precision of test results were carried out based on ISO 5725-2.

Based on the statistical results, three outliers were found within all the original test values. No specific outlying laboratory was found. It is believed that these outliers were caused by random errors.

The final precision value revealed by this statistical work could be used to determine the repeatability standard deviation and reproducibility standard deviation of this test method.

Meanwhile, the final precision value showed that the test method described in ISO/CD 18643 was reliable and showed consistency between the reported test values from all the participating laboratories.

Annex B
(informative)

Alternative method for determination of biuret in fertilizers

NOTE This method, using acetonitrile-free mobile phase (pure water), was submitted by experts from the Netherlands.[10] It was not incorporated in the international ring test (see Annex A). This method went through a three-sides (CHN-USA-NED) round robin test, in which the limitation of the method was elucidated and summarized as follows.

Summary of the method[10]:

Scope:

This acetonitrile-free HPLC method is suitable for determining biuret content within urea, some kinds of compound fertilizers, urea ammonium nitrate (UAN) solution. It is not suitable for the determination of the biuret content in urea formaldehyde slow release liquid fertilizer (e.g. Trisert®[2)]), without making changes to the conditions.

Mobile phase:

1 000 ml water. Filtered by 0,22 µm membrane as pre-treatment and ultrasonic degas for 10 min before used.

HPLC conditions:

Chromatographic column	Lichrospher RP-8 column, 4,6 × 250 mm, with 5 µm packing
Flow rate	1,0 ml/min
Inject volume	10 µl
Column temperature	30 °C
Detector wavelength	203 nm

2) Trisert® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Bibilography

[1] Mikkelsen R.L. Biuret in Urea Fertilizers. Better Crops. 2007, **91** (3) pp. 6–7

[2] Hojjatie M.M., & Abrams D. Validation for the determination of biuret in water-soluble urea-based commercial inorganic fertilizer materials, urea solutions, and sulfur-coated urea products by reverse-phase liquid chromatography: single-laboratory validation of an extension of AOAC Official Method 2003.14. J. AOAC Int. 2014, **97** (3) pp. 712–720

[3] AAPFCO Official Publication No. 67, 2014, Laws and Regulations

[4] Regulation (EC) No. 2003/2003

[5] GB/T 22924-2008, *Determination of biuret content for compound fertilizers (complex fertilizers)*

[6] GB/T 2441.2-2010, *Determination of urea — Part 2: Biuret content — Spectrophotometric method*

[7] AOAC 960.04, *Biuret in fertilizers — Spectrophotometric method*

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