International Seed Testing Association

Secretariat, Zürichstrasse 50, CH-8303 Bassersdorf, Switzerland
Phone: +41 44 838 60 00 Fax: +41 44 838 60 01
Email: ista.office@ista.ch - http://www.seedtest.org

Document 06-2010-OM

Method Validation Reports on proposed changes to the International Rules for Seed Testing 2011

Contents

ISTA validation study on seed viability testing of *Chloris gayana*

Stefanie Krämer[1] and Ronald Don[2]

[1] Landwirtschaftliches Technologiezentrum Augustenberg, Nesslerstrasse 23, 76227 Karlsruhe, Germany

[2] ISTA Secretariat, Zürichstrasse 50, 8303 Bassersdorf, Switzerland

September 2009

Summary

A validation study on seed viability testing of *Chloris gayana* using tetrazolium was carried out. Six laboratories were involved, and each tested 400 seeds of four seed lots. The results demonstrated that the method is of sufficient repeatability and reproducibility to be included in the ISTA Rules.

1. Plant material

Four seed samples of *Chloris gayana* of commercial quality were obtained by the Queensland Seed Technology Laboratory, Australia, for this study. The seeds were stored at 10 °C prior to distribution to participants.

a) Lot 1

b) Lot 2

c) Lot 3

d) Lot 4: This lot had a high content of empty seeds.

The samples were divided by the hand sampling method (ISTA Rules 2.5.2.2.4), and a purity test of 1 g was conducted on all samples prior to them being sent in December 2007. Lot 4 had a high content of empty seeds, but no attempt was taken to purify it and remove these. An in-house study by the Queensland Seed Technology Laboratory using 1000 seeds confirmed the homogeneity of the seed samples. Samples were sent to each of the participating laboratories in February 2009. The seeds were packed as blind samples (Lots 1–4).

2. Participating laboratories

Six laboratories from six countries participated in this validation study:

Mrs. Valerie Blouin**,** GEVES-SNES, B.P. 90024, 49071 Beaucouzé, France
e-mail: valerie.blouin@geves.fr

Mrs.Karen A. Hill, Queensland Seed Technology Lab, The University of Queensland, Gatton Campus, 4343 Queensland, Australia
e-mail: hillz1@bigpond.net.au

Mrs. Stefanie Krämer, Landwirtschaftliches Technologiezentrum Augustenberg, Nesslerstrasse 23, 76227, Karlsruhe, Germany
e-mail: stefanie.kraemer@ltz.bwl.de

Miss Linda Maile, NIAB, Official Seed Testing Station for England and Wales, Huntingdon Road, Cambridge CB3 0LE, United Kingdom
e-mail: linda.maile@niab.com

Mrs. Anny van Pijlen, General Netherlands Inspection Service (NAK), Randweg 14, Postbus 1115, 8300 BC Emmeloord, Netherlands
e-mail: apijlen@nak.nl

Mr. Garry Duffy, Seed Testing Laboratory, Backweston Lab. Campus, Young's Cross, Celbridge, Co. Kildare, Ireland
e-mail: Gary.Duffy@agriculture.gov.ie

In this report the laboratories are anonymously numbered as Laboratories 1–6; the sequence of these numbers is not identical to the alphabetical list given above.

3. Procedure for the TTC test

The testing method is described in Table 1, which is the proposal for inclusion in the ISTA Rules. Each laboratory tested 4 × 100 seeds from each of the 4 lots.

Table 1: Testing method for *Chloris gayana* as proposed for the ISTA Rules Change Proposals 2010

Species	Pretreatment: type/min. time (h)	Preparation before staining	Staining solution (%)	Optimum staining time (h)	Preparation for evaluation	Permitted non-viable tissue	Remarks
1	2	3	4	5	6	7	8
Chloris gayana	Remove glumes before premoistening. BP/16 at 10 °C; W/3	Cut transversely near embryo	1	6	Observe surface of embryo and scutellum	1/3 radicle, measured from radicle tip; in total 1/3 of extremities of scutellum	Empty seeds are reported as non-viable

4. Results

The results of the TTC viability tests were reported in April and July 2009. The results are given in Table 2 and shown in Figure 1.

Table 2: Seed viability (%) as reported for the four *Chloris gayana* seed samples by the 6 participating laboratories (results of the four replicates each containing 100 seeds)

	Seed viability (%)			
	Lot 1	Lot 2	Lot 3	Lot 4
Lab 1	77	67	64	26
	79	58	74	27
	76	63	72	29
	84	53	77	22
Lab 2	82	58	66	31
	67	65	60	34
	77	54	50	25
	90	58	65	19
Lab 3	83	60	69	27
	79	48	62	26
	74	55	56	30
	75	54	56	25
Lab 4	68	70	73	23
	67	65	70	24
	64	68	67	28
	66	61	68	23
Lab 5	73	64	87	61
	80	60	87	65
	75	70	86	65
	67	61	81	63
Lab 6	80	54	62	26
	76	61	63	31
	77	49	67	25
	83	50	71	20
Mean	76	59	69	32
95% confidence interval	±3	±3	±4	±6

The highest mean viability was 76 ± 3% for Lot 1, the lowest 32 ± 6% for Lot 4.

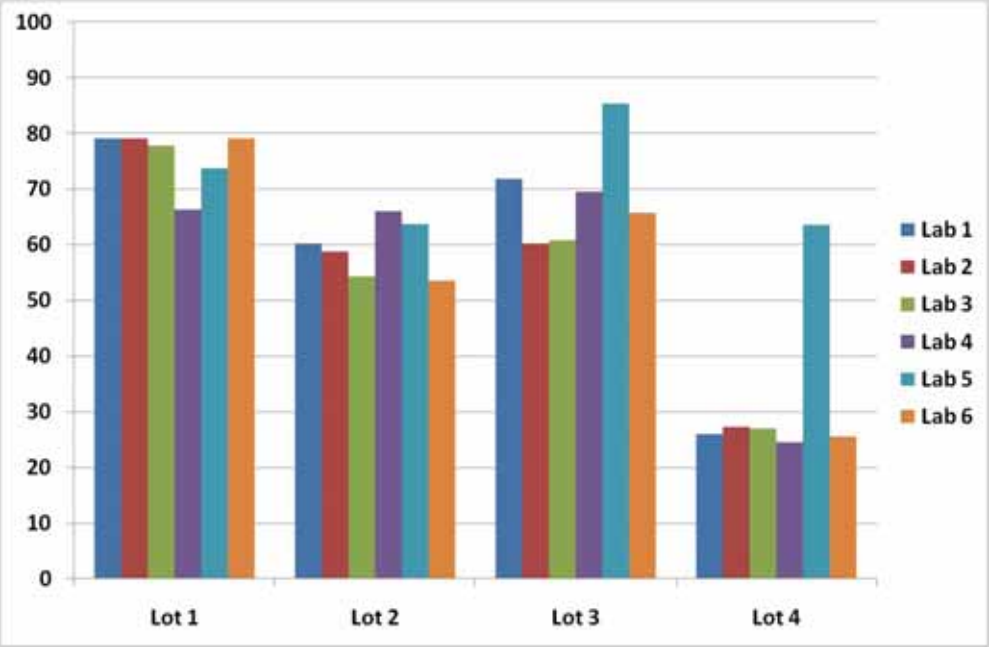

Figure 1: Viability test results for four *Chloris gayana* seed lots as reported by the six participating laboratories.

5. Statistical analysis

For statistical analysis the experimental error is quantified by the ratio f between the observed standard deviation (SD observed) and the expected standard deviation (SD expected) based on the binomial distribution:

$f = SD_{(obs.)} / SD_{(exp.)}$

$$SD_{(exp.)} = \sqrt{(p \times q)/n}$$

p: % TTC viability as mean;

q: 100 – p;

n = number of seeds.

Experimental error among the replicates

Table 3 shows the factors f for experimental error among the 4 replicates within a viability test in each of the 6 laboratories. The average factor f for 6 labs and 4 lots is 0.98, which is below 1.00.

Experimental error among tests in different laboratories

Table 4 shows the factors f for experimental errors among the 6 laboratories. The average factor f for 4 lots is 3.74. The individual f values for the lots are between 2.04 and 6.55. From Figure 1 it is clear that Laboratory 5 obtained a much higher viability for Lot 4 than other laboratories, and a plot of the mean viabilities obtained by the participating laboratories (Figure 2) demonstrates that Laboratory 5 obtained a higher mean viability. Analysis of the factor f experimental errors among participants when the results of Laboratory 5 are excluded show that the average factor f for the 4 lots is now 1.98, with individual f values ranging from 0.51 to 2.62.

Table 3: Experimental errors within the tests. The table shows for each combination of lot and laboratory the mean, the observed standard deviation between the 4 replicates, the expected standard deviation (based on the binomial distribution) and the f values

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Mean
Lot 1							
Mean	79	79	77.75	66.25	73.75	79	
SD observed	3.56	9.63	4.11	1.71	5.38	3.16	
SD expected	4.07	4.07	4.16	4.73	4.40	4.07	
f value	0.87	2.36	0.99	0.36	1.22	0.78	1.10
Lot 2							
Mean	60.25	58.75	54.25	66	63.75	53.5	
SD observed	6.08	4.75	4.92	3.92	4.50	5.45	
SD expected	4.89	4.92	4.98	4.74	4.81	4.99	
f value	1.24	0.93	0.99	0.83	0.94	1.09	1.00
Lot 3							
Mean	71.75	60.25	60.75	69.50	85.25	65.75	
SD observed	5.56	7.32	6.18	2.65	2.87	4.11	
SD expected	4.50	4.89	4.88	4.60	3.55	4.75	
f value	1.24	1.50	1.27	0.57	0.81	0.87	1.04
Lot 4							
Mean	26.00	27.25	27.00	24.50	63.50	25.50	
SD observed	2.94	6.65	2.16	2.38	1.91	4.51	
SD expected	4.39	4.45	4.44	4.30	4.81	4.36	
f value	0.67	1.49	0.49	0.55	0.40	1.03	0.77

Table 4: Experimental errors between the laboratories. The table shows for each lot the mean, the observed standard deviation (SD), the expected standard deviation (based on the binomial distribution) and the f values

Lot	Mean viability (%)	Observed SD (%)	Expected SD (%)	f value
Lot 1	80	5.10	2.14	2.38
Lot 1 without Lab 5	76	5.59	2.13	2.62
Lot 2	59	5.00	2.46	2.04
Lot 2 without Lab 5	59	5.06	2.46	2.62
Lot 3	69	9.24	2.32	3.99
Lot 3 without Lab 5	66	5.13	2.38	2.16
Lot 4	32	15.32	2.34	6.55
Lot 4 without Lab 5	26	1.12	2.19	0.51
Mean all labs				3.74
Mean without Lab 5				1.98

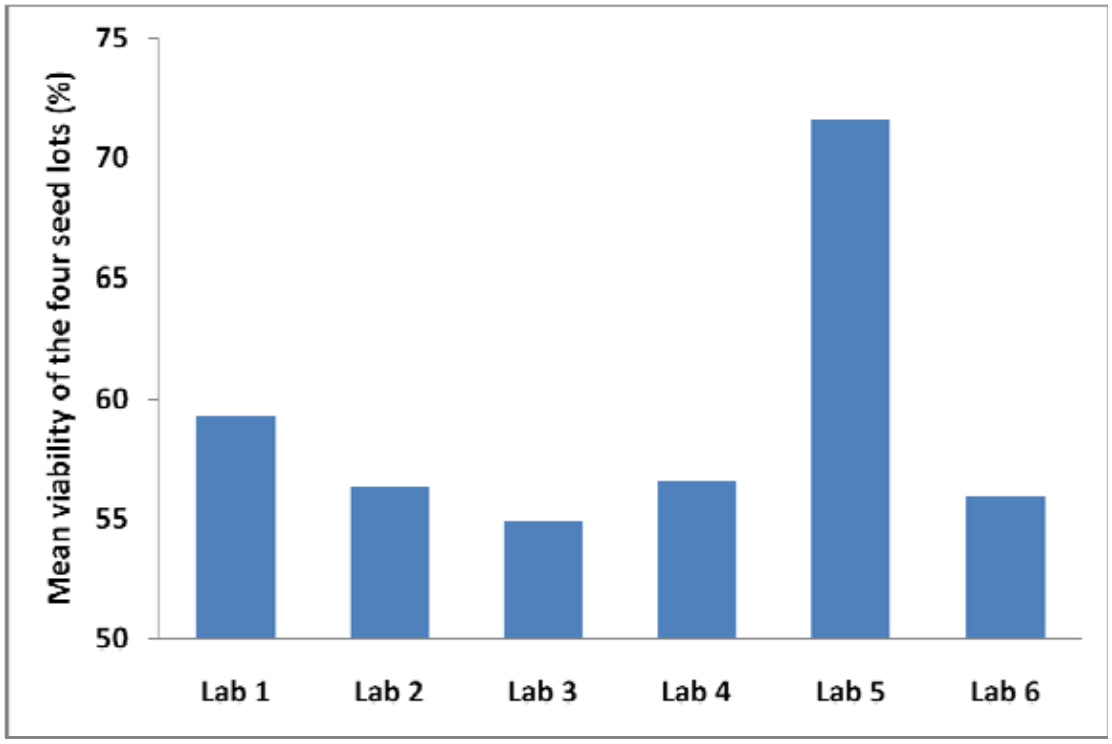

Figure 2: Mean viability test results for *Chloris gayana* seed lots as reported by the six participating laboratories

The f value used for establishing the tolerance tables for seed viability test results in the ISTA Rules is 2.82. Thus, even when including the results of Laboratory 5, the average f factor of 3.74 indicates a high but still acceptable experimental error among tests in different laboratories. When the results of Laboratory 5 are excluded, the average f factor of 1.98 indicates a totally acceptable experimental error among tests in different laboratories.

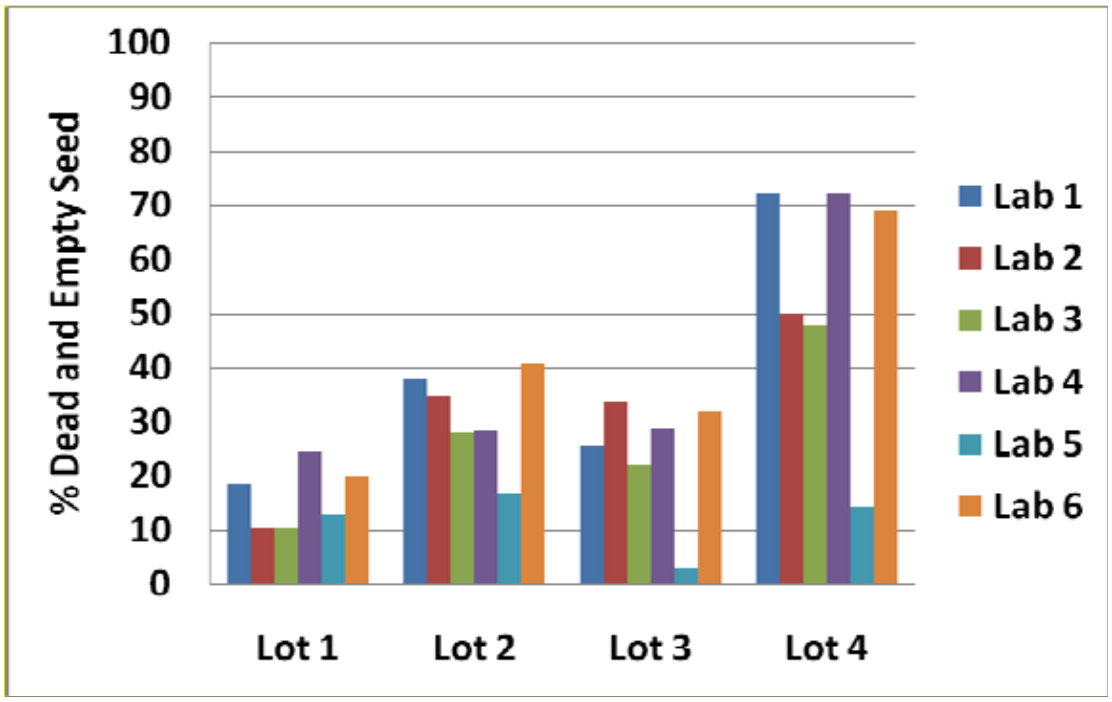

Figure 3: The levels of dead and empty seeds found in the 4 seed lots of *Chloris gayana* by the six participating laboratories

The reason for Laboratory 5 reporting higher results is that this laboratory was the only one of the participants to purify the seeds prior to carrying out the tetrazolium test. All other participants carried out the test on the seeds as received, without making any attempt to remove empty seeds. Because of this, Laboratory 5 reported fewer dead or empty seeds than the others (Figure 3).

As a further test, the maximum tolerated ranges for the mean viabilities were calculated using the formula S = f × SD × F, as according to Miles (1963). This test was performed with and without the results of Laboratory 5. In only one case (Lot 4 including Laboratory 5) did the actual range exceed the tolerated range, and it did so by less than 2%. Thus, the range as a further measure indicates that the experimental error is acceptable.

Table 5: Maximum tolerated ranges S according to Miles (1963)

Lot	S (%)	Mean	f value	SD expected	F	Actual range
All Lot 1	33.9	72	2.82	2.13	5.62	12.75
All Lot 2	38.9	59	2.82	2.46	5.62	12.50
All Lot 3	36.7	69	2.82	2.32	5.62	25.00
All Lot 4	37.1	32	2.82	2.34	5.62	39.00
Lot 1 without lab 5	32.8	76	2.82	2.13	5.46	12.75
Lot 2 without lab 5	37.9	59	2.82	2.46	5.46	12.50
Lot 3 without lab 5	36.6	66	2.82	2.38	5.46	11.50
Lot 4 without lab 5	33.8	26	2.82	2.19	5.46	2.75

6. Conclusion

The f factors in Table 3 indicate an acceptable experimental error among the 4 replicates within the tests. Moreover, the maximum tolerated ranges in Table 5 indicate acceptable variation between participating laboratories. Laboratory 5 was the only participant to attempt purification of the samples prior to tetrazolium testing, and the results are even more impressive if the results from Laboratory 5 are excluded from the analysis. Thus, there is no reason to assume that the procedure given in Table 1 should not be introduced into the ISTA Rules.

Proposal to change the duration of the drying period for *Lolium* spp. for the high-temperature oven method from 1 to 2 hours

Craig McGill

Summary

The PT round 08-1 included moisture determination of *Lolium multiflorum*. The results of this round indicated there was a difference in the moisture determined for *Lolium multiflorum* depending on which of the two methods (103 °C for 17 hours or 130 °C for 1 hour) permitted in the ISTA Rules (2009) was used. A comparative testing study was undertaken by four ISTA laboratories to determine whether moisture determination in *Lolium multiflorum* at 130 °C for 1, 2 or 3 hours gave the same result as the reference method (17 hours at 103 °C). The data from this comparative testing supports the proposal that the duration of the moisture test for *Lolium* spp. be increased from 1 to 2 hours in the ISTA Rules (Table 9A Part 1):

Species	Grinding/cutting (9.1.5.4, 9.1.5.5)	High temperature	Drying at high temperature (h)	Predrying requirement (9.1.5.6)
1	2	3	4	5
Lolium spp.	No	Yes	2	–

Introduction

In 2008 a new reference method was adopted for moisture testing (ISTA Rules, 2009). The new reference method is the low-temperature constant oven method, i.e. 17 hours at 103 °C. The low-temperature constant oven method can be used for all species in Table 9 of the ISTA Rules. The high-temperature constant oven method can be used as an alternative method where indicated in Table 9. The PT round 08-1 included moisture testing of *Lolium multiflorum*. This was the first time that alternate methods could be used. The results of the PT round 08-1 indicated a difference in the moisture result for *Lolium multiflorum,* depending on the method used (Table 1). These results alone are not sufficient to support a change in the high-temperature constant oven method for *Lolium* spp. However, a comparative testing round was undertaken by four ISTA laboratories, under the leadership of LaRAS, to determine whether the moisture contents determined by the low-temperature constant oven method and the high-temperature constant oven method for *Lolium* spp. are the same. The results of this comparative testing are the basis of this validation report.

Table 1. Moisture content determined in *Lolium perenne* seed lots in ISTA PT round 08 using the low-temperature and high-temperature oven methods

Seed lot	Oven method	
	Moisture after 17 h at 103 °C (%) (average of 6 labs)	Moisture after 1 h at 130 °C (%) (average of 100 labs)
1	10.7	9.6
2	11.6	10.7
3	15.1	14.2

Materials and methods

Two seed lots of *Lolium multiflorum* were evaluated. These were the same seed lots used in PT round 08-1 and were obtained from the test organiser for moisture determination in PT round 08-1.

Four ISTA-accredited laboratories from three countries participated in the comparative testing:

ISTA laboratory	Contact person	Accredited for moisture determination
Ente Nazionale Sementi Elette, Laboratorio Analisi Sementi [ITDL0300]	Rita Zecchinelli	Yes
Forschungsanstalt Agroscope Reckenholz-Tänikon ART [CHDL0100]	Silvia Zanetti	Yes
GEVES (Station Nationale d'Essais de Semences [FRDL0200])	Maria Rosaria Mannino	Yes
LaRAS (Laboratorio di Ricerca e Analisi Sementi [ITDL0100])	Enrico Noli	Yes

The low-temperature and high-temperature methods were followed as indicated in the ISTA Rules, with duplicate determinations carried out on each sample.

Samples were distributed to the laboratories in sealed (moisture-proof) aluminium packets. The laboratories were instructed to begin the moisture determination immediately after the packets were opened, and that all samples should be tested at the same time, i.e. only one experiment at 101–105 °C and one at 130–133 °C.

The moisture of the samples was determined in the following ways:

High-temperature oven method

The moisture of the samples was first determined using the high-temperature oven method as described in Chapter 9.1 of the ISTA Rules (2009). At the end of the prescribed drying period (1 hour at 130 °C), samples were allowed to cool and then weighed. Samples were then returned to the oven for a further 1 hour's drying. At the end of the second hour of drying, samples were again allowed to cool before reweighing and were then returned to the oven for a further 1 hour's drying. Samples were again allowed to cool before reweighing.

Low-temperature oven method

The moisture of the samples was first determined using the low-temperature oven method as described in Chapter 9.1 of the ISTA Rules (2009). At the end of the prescribed drying period (17 hours at 103 °C), samples were allowed to cool and then weighed. Samples were then returned to the oven for a further 2 hours' drying. At the end of the second two-hour drying period, samples were again allowed to cool before reweighing. The second two-hour drying period was based on ISTA Rule 9.1.4.2 (ISTA Rules, 2009) for checking the ventilation of the oven.

All drying periods were begun when the oven had returned to the set temperature.

Data analysis

The reference method for moisture determination is 17 hours at 103 °C. However, a shorter determination at 130 °C may be used if properly validated. A tolerance of 0.3% is permitted for the comparison between the reference method and a shorter duration test at 130 °C. The shorter-duration 130 °C method is accepted if 75% or more of the differences between the mean of the two replicates for each method are within the tolerated range of ±0.3% (ISTA, 2007). This tolerance

was used in this validation study to compare the moisture determinations for each sample by each laboratory at 103 °C for 17 hours with 103 °C for 19 hours and 130 °C for 1, 2 and 3 hours.

To investigate the interactions between different laboratories, samples, temperatures and duration, the data was subjected to an analysis of variance (ANOVA). A general liner model (GLM) was used to determine significant interactions between treatments. Where significant effects were detected in the ANOVA (P = 0.05), means were compared using the Tukey test. Prior to analysis, data were checked for normality using the univariate procedure in SAS (Release 8.2 (TS2M0), SAS Institute Inc., Cary, NC, USA). No transformation of the data was necessary.

Results and discussion

The recovery times for the moisture ovens used by the four laboratories are given in Table 2.

Table 2. Time taken for the moisture ovens used in the comparative testing to return to 103 °C or 130 °C

Laboratory	Time taken (minutes) for the oven to return to the set temperature	
	High-temperature method (130 °C)	Low-temperature method (103 °C)
Laboratory 1	5 to 15	4 to 5
Laboratory 2	30	25
Laboratory 3	5 to 15	4 to 5
Laboratory 4	4	2 to 3

Recovery times for the ovens are all with the limit prescribed in the ISTA Rules (9.1.4.2).

The results of the moisture determinations of the four laboratories and the difference in the moisture determinations from each laboratory are given in Tables 3–6.

Table 3. Comparison between the moisture determined for two seed lots of *Lolium multiflorum* at 103 °C for 17 hours (low-temperature (reference) oven method) with that determined using the high-temperature oven method of 130 °C for 1 hour. Samples with a difference in moisture content of ±0.3% or greater are out of tolerance.

Laboratory	Sample	Reference method moisture (%)	Moisture (%) determined after 1 hour at 130 °C	Difference between the two methods (%)	In tolerance (±0.3%)
1	1	11.37	10.77	0.40	No
2	1	11.37	11.23	0.14	Yes
3	1	11.47	11.10	0.37	No
4	1	11.49	11.04	0.45	No
1	2	11.59	10.67	0.08	Yes
2	2	11.63	11.04	0.59	No
3	2	11.76	11.02	0.74	No
4	2	11.78	10.92	0.86	No

Only two (25%) of the moisture determinations were in tolerance.

Table 4. Comparison between the moisture determined for two seed lots of *Lolium multiflorum* at 103 °C for 17 hours (low-temperature (reference) oven method) with that determined using the high-temperature oven method of 130 °C for 1 hour, plus an extra 1 hour drying. Samples with a difference in moisture content of ±0.3% or greater are out of tolerance.

Laboratory	Sample	Reference method moisture (%)	Moisture (%) determined after 1 hour at 130 °C + 1 hour at 130 °C	Difference between the two methods (%)	In tolerance (±0.3%)
1	1	11.37	11.38	0.01	Yes
2	1	11.37	11.59	0.22	Yes
3	1	11.47	11.54	0.07	Yes
4	1	11.49	11.55	0.06	Yes
1	2	11.59	11.43	0.16	Yes
2	2	11.63	11.60	0.03	Yes
3	2	11.76	11.67	0.09	Yes
4	2	11.78	11.61	0.17	Yes

All moisture determinations were in tolerance.

Table 5. Comparison between the moisture determined for two seed lots of *Lolium multiflorum* at 103 °C for 17 hours (low-temperature (reference) oven method) with that determined using the high-temperature oven method of 130 °C for 1 hour, plus an extra 2 hours drying. Samples with a difference in moisture content of ±0.3% or greater are out of tolerance.

Laboratory	Sample	Reference method moisture (%)	Moisture (%) determined after 1 hour at 130 °C + 1 hour + 1 hour at 130 °C	Difference between the two methods (%)	In tolerance (±0.3%)
1	1	11.37	11.61	0.24	Yes
2	1	11.37	11.70	0.33	No
3	1	11.47	11.73	0.29	Yes
4	1	11.49	11.76	0.27	Yes
1	2	11.59	11.73	0.14	Yes
2	2	11.63	11.78	0.15	Yes
3	2	11.76	11.85	0.09	Yes
4	2	11.78	11.90	0.12	Yes

Seven out of eight (87.5%) moisture determinations were in tolerance.

Table 6. Comparison between the moisture determined for two seed lots of *Lolium multiflorum* at 103 °C for 17 hours (low-temperature (reference) oven method) with that determined using the low-temperature (reference) oven method of 103 °C for 17 hours, plus an extra 2 hours drying. Samples with a difference in moisture content of ±0.3% or greater are out of tolerance.

Laboratory	Sample	Reference method moisture (%)	Moisture (%) determined after 17 hours at 103 °C + 2 hours at 103 °C	Difference between the two methods (%)	In tolerance (±0.3%)
1	1	11.37	11.37	0	Yes
2	1	11.37	11.42	0.05	Yes
3	1	11.47	11.52	0.05	Yes
4	1	11.49	11.47	0.02	Yes
1	2	11.59	11.62	0.03	Yes
2	2	11.63	11.69	0.06	Yes
3	2	11.76	11.80	0.04	Yes
4	2	11.78	11.78	0	Yes

All moisture determinations are in tolerance.

The ANOVA table (Table 7) indicates that there were significant differences in moisture determination between the laboratories (Table 8), duration (Table 9) and seed lots. The moisture content of the seed lots was 11.54% and 11.42% (minimum significant difference ($P < 0.05$) = 0.044). While this difference may be statistically significant, the actual difference is very small – less than the difference that would be acceptable for duplicate determinations on the same sample (ISTA Rule 9.1.6.2; ISTA Rules, 2009). In practical terms, therefore, this difference is not important.

Table 7. ANOVA table for moisture determination in two lots of *Lolium multiflorum*

Source	DF	Type I SS	Mean square	F value	Pr > F
Laboratory	3	0.233	0.078	16.71	<0.0001
Seed lot	1	0.143	0.143	30.72	<0.0001
Temperature	1	0.201	0.201	43.29	<0.0001
Duration	3	2.634	0.878	188.85	<0.0001
Seed lot · temperature	1	0.154	0.154	33.02	<0.0001
Seed lot · temperature · duration	3	0.062	0.021	4.48	0.0112

Table 8. Moisture content determined by all laboratories for both seed lots at all temperatures and durations.

Laboratory	Moisture content (%)
1	11.35
2	11.51
3	11.55
4	11.53
Minimum significant difference (P < 0.05)	0.0834

The ANOVA indicates that the moisture content determined by Laboratory 1 differs from that determined by laboratories 2, 3 and 4. The difference in the moisture content determined by the different laboratories is 0.2% or less. There are no tolerance tables for comparing moisture determinations between laboratories however a tolerance of 0.2% is used for comparisons between duplicate moisture determinations performed in the same laboratory at the same time on the same sample (ISTA Rule 9.1.6.2; ISTA, 2009). It is not unreasonable to expect that, because of the increased sources of error, a tolerance calculated for the difference in moisture determinations on the same sample in different laboratories would be greater than 0.2%. Therefore while there may be a statistically significant difference in the moisture determination between laboratory 1 and laboratories 2, 3 and 4, the actual difference is very small, and there is no reason to remove the results from laboratory 1 from the analysis.

Table 9. Moisture content determined by all laboratories for all seed lots for different drying durations.

Test method	Moisture content (%)
103 °C for 17 hours	11.56
103 °C for 19 hours	11.58
130 °C for 1 hour	10.97
130 °C for 2 hours	11.55
130 °C for 3 hours	11.76
Minimum significant difference (P < 0.05)	0.100

There was no significant difference in the moisture determined at 103 °C for 17 hours or 19 hours or 130 °C for 2 hours. The moisture content determined after 1 hour drying at 130 °C was significantly lower than that determined for any other method. Similarly moisture content determined after 3 hours drying at 130 °C was significantly higher than that determined for any other method.

There was also a significant interaction effect between the sample, the temperature at which the moisture determination was performed and the duration of the moisture determination (Table 7, Figure 1).

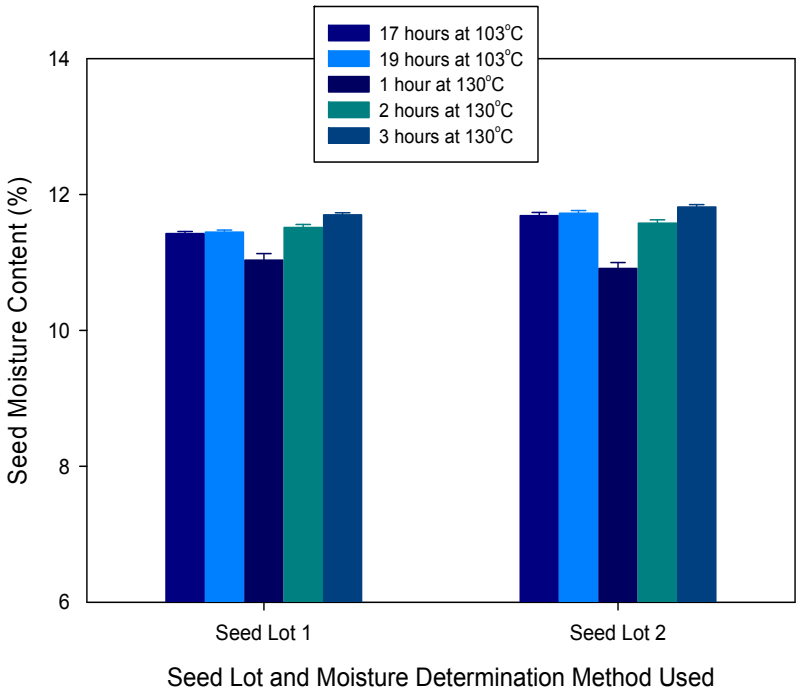

Figure 1. Moisture content (%) determined in two seed lots of *Lolium multiflorum* using the low-temperature oven method or the high-temperature oven method for different durations. Error bars are the standard error of the mean for each moisture determination method.

For both seed lots there was a significant difference in the moisture determined using 103 °C for 17 hours compared to 130 °C for 1 hour with more water being lost after 17 hours at 103 °C. This suggests that 1 hour at 130 °C is insufficient to remove all the moisture from *Lolium multiflorum*.

There was no significant difference in the moistures determined using 103 °C for 17 hours and 130 °C for 2 hours, suggesting that a drying duration of 2 hours is more appropriate for *Lolium multiflorum*. The results for 3 hours drying at 130 °C are less clear. For sample one significantly more weight was lost after 3 hours drying at 130 °C than after 17 hours drying at 103 °C, but not for the second sample. The data is therefore inconclusive as to whether more water is being lost after three hours at 130 °C.

There was no significant difference in the moisture content determined when the duration of the low-temperature method was extended from 17 to 19 hours confirming that after 17 hours at 103 °C no further moisture is lost from *Lolium multiflorum*.

The comparative testing has been performed using *Lolium multiflorum*. Table 9 Part 1 (ISTA Rules, 2009) does not distinguish between species of *Lolium*. An assumption made in this validation is that moisture determination on other species of *Lolium* would give similar results. There is experimental evidence available to support this assumption. Grabe (1984) presents data that indicates that moisture determination in *Lolium perenne* using 17 hours at 103 °C and 2 hours at 130 °C gives similar results.

Limitations of the validation study

A limitation of this validation study is that one moisture content only was used to compare the high-temperature and low-temperature oven methods. There is data published (Benjamin & Grabe 1988) that indicates there is no single drying period at 130 °C that gives an accurate moisture determination over a range of moisture contents in *Lolium perenne,* i.e. 6 hours for whole seed at around 6% moisture, 3 hours at around 9% and 2 hours at around 15%. Different drying durations

for seed at different moisture levels are not practical, as they require prior knowledge of the seed moisture content. Table 10 gives the percentage of moisture lost (at 130 °C) from *Lolium perenne* as a percentage of the total moisture in the seed. In contrast to Benjamin & Grabe (1988), these data suggest that six hours' drying is required to remove 100% of the moisture, including samples with high moisture content. Nonetheless, the data do show that one hour is too short a drying duration, and that two hours may be a good compromise.

Table 10. Percentage of moisture of total lost (at 130 °C) over time as influenced by absolute moisture level for *Lolium perenne* (Nijënstein, n.d.)

Moisture content: level after 6 hours. Percentage of total lost over time (minutes)					
Moisture content	15	30	60	120	360
12.37	60	81	89	95	100
16.92	76	85	92	97	100
20.70	73	86	95	97	100
23.91	82	89	94	97	100
27.64	84	91	95	98	100
30.50	78	91	96	97	100
32.56	71	91	96	98	100
35.04	69	93	97	99	100
39.34	65	90	97	99	100

Conclusions and Recommendations

Only one moisture content level was used to compare the high-temperature and low-temperature oven methods; therefore, the study may not have given a clear/correct drying period. Nonetheless, the data from PT round 08-1 (Table 1) and this validation study have demonstrated that the one-hour duration for the high-temperature oven method is too short to accurately determine the moisture content in *Lolium multiflorum,* and that a change from this duration is required immediately. Previously published data suggest that no single drying period at 130 °C gives an accurate moisture determination over a range of moisture contents in *Lolium* spp., but that 2 hours may be a good compromise. The PT round 08-1, this validation study and the literature combined provide evidence to support the recommendation that the duration of the high-temperature oven method for *Lolium* spp. be increased to two hours.

Acknowledgements

The Moisture Committee would like to thank Enrico Noli form LaRAS for initiating the comparative testing that forms the basis of this validation study, and the four laboratories, LaRAS, ENSE Agroscope and GEVES for participating in the comparative testing.

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Proposal for the addition of *Glycine max* as a species to which the conductivity test for seed vigour can be applied.

Alison A. Powell
School of Biological Sciences, University of Aberdeen, 23, St Machar Drive, Aberdeen, AB24 3UU, UK

a.a.powell@abdn.ac.uk

Summary

Five seed lots of *Glycine max*, all having a laboratory germination of >80%, were tested by seven laboratories using the electrical conductivity test, as described in the ISTA Rules for *Pisum sativum*. All laboratories consistently identified the same significant differences in the seed lot conductivity and the data was repeatable within laboratories and reproducible between laboratories. The results of all tests gave a z-score between +2.00 and –2.00 and all data fell within the tolerance levels established for peas in the ISTA Rules. This provides evidence in support of the inclusion of *Glycine max* within the ISTA Rules as a species to which the conductivity test can be applied.

Introduction

The conductivity test is currently validated in the ISTA Rules as a test that can be applied to *Pisum sativum.* In 2008, the test was also validated for application to *Phaseolus vulgaris* (see Method Validation Report*)* and the addition of *P. vulgaris* to the ISTA Rules as a species to which the conductivity test can be applied is a Rules Proposal for 2010. The basis of the conductivity test is the solute leakage from seeds into water. The extent of solute leakage can be attributed to impaired membrane integrity and the development of dead tissue on the living cotyledons as the result of seed ageing or imbibition damage (Matthews and Powell, 2006), both of which are common to most grain legumes (Powell, Matthews and Oliveira, 1984). It is therefore not surprising that measurements of solute leakage, using the conductivity test, identified differences in the vigour of soya bean (*Glycine max*) seed lots, as reflected in their field emergence (Oliveira *et al*., 1984*;* Yaklich *et al.*, 1979). The aim of this study was to demonstrate that the conductivity test applied to *Glycine max* is both repeatable within laboratories and reproducible between laboratories.

Materials and Methods

Samples of five seed lots of *Glycine max* were supplied by Rasha El-Khadem, from Pioneer HiBred, Austria. The seeds originated from Italy and had standard germinations above 80%. Coded samples of the seed lots were sent from Aberdeen UK to the participating laboratories, namely SNES, GEVES, Angers, France; LaRAS, Bologna, Italy; OSTS, SASA, Edinburgh, UK; OSTS, NIAB, Cambridge, UK; Department of Horticulture, Ege University, Izmir, Turkey; Department of Crop Science, University of Ferdowsi, Mashhad, Iran; Seminis, Enkhuizen, The Netherlands. The participants in the test were limited to those in countries to which the soyabean seeds could be readily exported.

Each laboratory completed the conductivity test using the same method as that described for peas in the ISTA Rules (ISTA, 2009) i.e. 4 replicates of 50 seeds, each soaked in 250 ml deionised/ distilled water for 24 h at 20 °C.

The data was analysed using (a) Analysis of Variance, (b) calculation of z-scores and (c) the statistical tool developed by S. Grégoire according to ISO 5725-2 and available for download at the ISTA website: http://www.seedtest.org/upload/cms/user/ISO572511.zip

Results

The seed lot means (Table 1) revealed clear and significant differences in seed leachate conductivity and hence vigour. Seed lot E had the highest conductivity (23.5 µS cm^{-1} g^{-1}), that is the lowest vigour, followed by lot D, lots A and B (not significantly different from each other) and lot C (16.21 µS cm^{-1} g^{-1}, highest vigour). Lots E and D were consistently identified as having the highest conductivity (lowest vigour) in every laboratory (Table 1), while lot C always had the lowest conductivity (highest vigour). Application of the tolerance tables from Chapter 15 of the ISTA Rules (ISTA, 2009) revealed that, the replicate data (Appendix 1) for each lot in each laboratory were in tolerance with one another, as were the test results for each lot from the seven different laboratories. There were small, but significant, differences in the overall means from the seven laboratories (Table 1). The coefficient of variation for the comparative test was 6.4%, a value comparable with that reported (4.3%) for the method validation of conductivity for *Phaseolus vulgaris* (Powell, 2009).

Calculation of the z-scores (Table 2) revealed that all data fell within the values +2.0 to –2.0 that are acceptable within ISTA proficiency tests

Repeatability and reproducibility were analysed with the statistical tool developed by S. Grégoire, based on ISO 5725-2; this allows the calculation of h- and k-values. The h-values show the tendency for a laboratory to give over-estimations or under-estimations compared to the mean of all the results available whereas the k-values give a measure of the variability of the repeats. Higher values indicate greater under- or over-estimations (h-values) or greater variability between replicates (k-values).

There was only one significant h-value, namely for lot 2, in lab 4 (Figure 1) which indicated that the result was significantly overestimated. Significant k values were found for two lots in each of two laboratories (lab 3, lots 1 and 2; lab 6, lots 3 and 4), indicating that there was greater variability between replicates. Even so, the replicates were in tolerance (Appendix 1; Chapter 15, ISTA Rules, [ISTA 2009]).

Repeatability and reproducibility values are affected by the seed quality of the lots tested, with low vigour seeds often having higher values. It is therefore not possible to compare directly the data from comparative tests using different seed lots. However, the values obtained for soya bean (Table 3) were similar to and lower than values previously obtained for *Phaseolus vulgaris* (repeatability: 0.9511–2.2287; reproducibility: 1.6850–4.2581).

Discussion

The conductivity test consistently identified differences between seed lots in each of seven laboratories. The test was both repeatable within laboratories and reproducible in different laboratories. In addition, the replicates within the laboratories and the mean values obtained for each lot in different laboratories all fell within tolerance, using the tolerance tables in the ISTA Rules (ISTA, 2009). This provides evidence in support of the addition of *Glycine max* to the ISTA Rules as a species for which the conductivity test can be applied.

Acknowledgements:

I am very grateful to Rasha El-Khadem, from Pioneer Hi Bred, Parndorf, Austria, for supplying the seeds, and to Emanuela Casarini, Hulya Ilbi, Mohammad Khajeh Hosseini, Tim Loeffler, Gillian McLaren, Jane Taylor and Marie-Hélène Wagner for participating in this comparative test.

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Table 1. Comparison of laboratory and seed lot means of five seed lots of soya beans tested by seven laboratories using the conductivity test

Lab	**Lot**					**Lab means**
	A	**B**	**C**	**D**	**E**	
1	18.9 ^{C}	17.4 ^{D}	15.3 ^{F}	21.2 ^{B}	22.2 ^{A}	19.0 ^{d}
2	18.6 ^{B}	17.8 ^{B}	16.4 ^{C}	21.8 ^{A}	22.7 ^{A}	19.4 ^{cd}
3	18.5 ^{D}	19.4 ^{C}	17.1 ^{E}	21.5 ^{B}	23.0 ^{A}	19.9 ^{bc}
4	18.9 ^{C}	20.8 ^{B}	16.3 ^{D}	21.4 ^{B}	25.1 ^{A}	20.5 ^{ab}
5	15.8 ^{D}	18.0 ^{C}	15.8 ^{D}	20.6 ^{B}	23.9 ^{A}	18.8 ^{d}
6	19.3 ^{C}	19.3 ^{C}	17.4 ^{D}	22.8 ^{B}	26.3 ^{A}	21.0 ^{a}
7	16.0 ^{CB}	16.8 ^{B}	15.3 ^{C}	20.2 ^{A}	20.9 ^{A}	17.8 ^{e}
Lot means	18.0 ^{c}	18.5 ^{c}	16.21 ^{d}	21.4 ^{b}	23.5 ^{a}	

For lot and lab means, different lower-case letters indicate that values are significantly different using LSD at the 5% level

Within a row (laboratory), different upper-case letters indicate that values (lots) are significantly different using LSD at the 5% level

Table 2: Comparison of means, standard deviations (SD) and z-scores for five seed lots of soya beans tested by seven laboratories using the conductivity test

	Lot				
Lab	A	B	C	D	E
a) means					
1	18.9	17.4	15.3	21.2	22.2
2	18.6	17.8	16.4	21.8	22.7
3	18.5	19.4	17.1	21.5	23.0
4	18.9	20.8	16.4	21.4	25.1
5	15.8	18.0	15.8	20.6	23.9
6	19.3	19.3	17.4	22.8	26.3
7	16.0	16.8	15.3	20.2	20.9
Mean	17.99	18.47	16.21	21.37	23.46
SD	1.463	1.373	0.827	0.836	1.822
b) z-scores					
1	0.62	-0.78	-1.10	-0.60	-0.69
2	0.39	-0.52	0.17	0.53	-0.43
3	0.38	0.65	1.12	0.19	-0.23
4	0.59	1.66	0.06	0.08	0.92
5	-1.50	-0.34	-0.50	-0.92	0.24
6	0.91	0.57	1.39	1.71	1.57
7	-1.37	-1.22	-1.15	-1.36	-1.38

Table 3: Values for repeatability and reproducibility of results from the conductivity test on *Glycine max*

Lot	Repeatability	Reproducibility
A	1.0097	1.7318
B	1.2622	1.8014
C	0.7503	1.0589
D	1.1318	1.2908
E	1.8131	2.4901

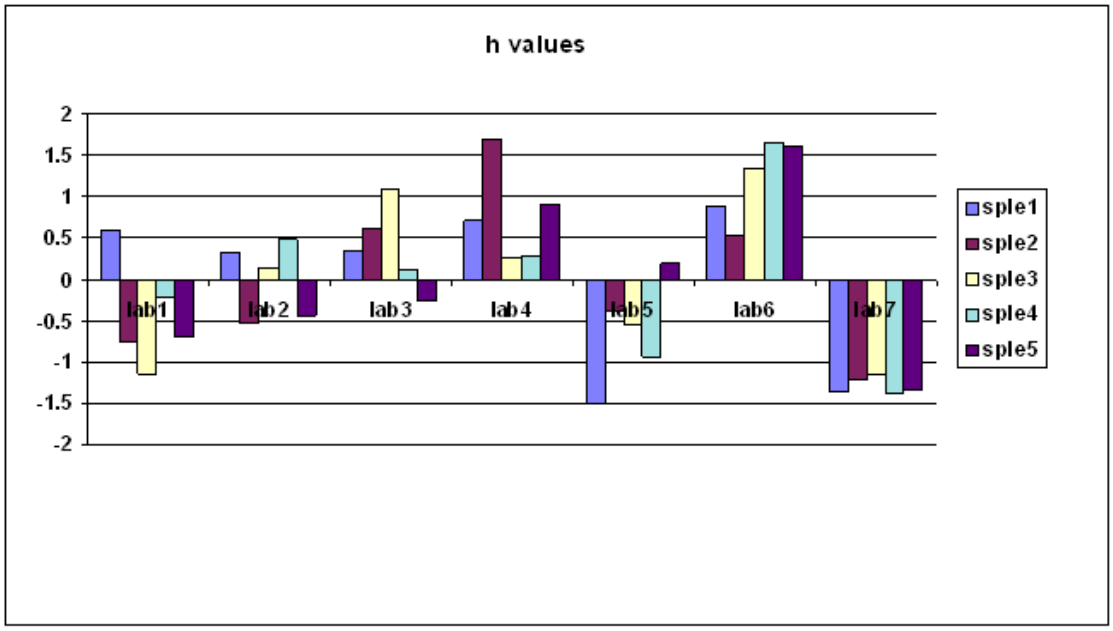

Figure 1: h-values for five seed lots of *Glycine max* tested using the conductivity test in seven laboratories.

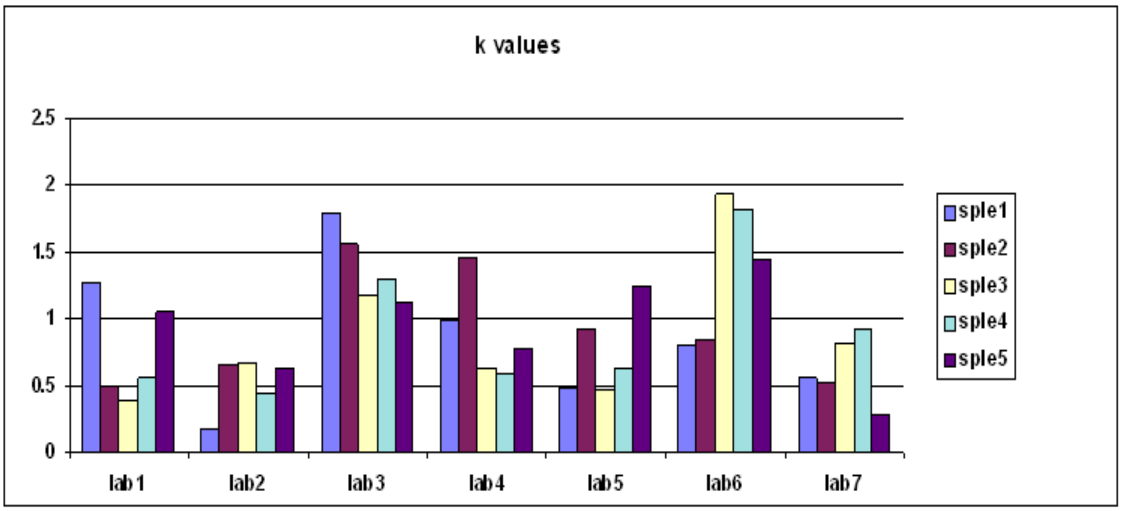

Figure 2: k-values for five seed lots of *Glycine max* tested using the conductivity test in seven laboratories.

Appendix 1: Data for each replicate conductivity reading for each of five lots taken in each of seven laboratories

Lot	Replicate	Lab						
		1	**2**	**3**	**4**	**5**	**6**	**7**
A	1	17.44	18.31	20.44	18.01	16.22	18.33	15.84
	2	19.11	18.87	16.31	18.17	15.06	19.16	15.28
	3	20.49	18.4	17.91	19.11	16.04	20.17	16.56
	4	18.67	18.64	19.51	20.16	15.78	19.67	16.24
	Mean	**18.93**	**18.56**	**18.54**	**18.86**	**15.8**	**19.33**	**15.98**
B	1	16.58	18.73	18.38	22.58	19.75	17.82	17.32
	2	17.38	16.72	22.17	21.5	17.28	20	16.22
	3	17.60	17.68	17.77	20.62	17.61	19.09	17.39
	4	18.11	17.93	19.11	18.3	17.31	20.14	16.22
	Mean	**17.41**	**17.76**	**19.36**	**20.75**	**17.99**	**19.26**	**16.79**
C	1	15.29	16.5	17.57	16.43	15.60	17.14	15.29
	2	15.58	16.02	16.13	16.11	15.56	18.27	14.46
	3	14.95	15.86	18.11	15.67	15.59	15.45	15.95
	4	15.27	16.99	16.75	16.82	16.26	18.6	15.34
	Mean	**15.27**	**16.35**	**17.14**	**16.26**	**15.75**	**17.36**	**15.26**
D	1	21.27	21.52	19.71	22.25	20.72	21.74	20.09
	2	21.91	21.34	23.24	20.77	19.82	20.53	18.91
	3	21.31	22.38	21.93	21.07	20.41	24.88	20.51
	4	20.35	21.99	21.22	21.67	21.54	24.05	21.4
	Mean	**21.21**	**21.81**	**21.53**	**21.44**	**20.62**	**22.8**	**20.23**
E	1	20.85	23.67	25.15	26.22	22.86	27.37	20.91
	2	25.05	21.08	21.45	25.79	21.83	22.8	21.59
	3	21.81	22.64	24.47	23.1	27.02	28.5	20.35
	4	21.20	23.34	21.1	25.45	23.93	27.85	20.94
	Mean	**22.23**	**22.68**	**23.04**	**25.14**	**23.91**	**26.32**	**20.94**