CPVO · OCVV
Community Plant Variety Office
Office Communautaire des Variétés Végétales

PROTOCOL FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTS

Hordeum vulgare L.

BARLEY

UPOV Code: HORDE_VUL

Adopted on 19/03/2019

Entered into force on 01/08/2019

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CPVO/TP-019/5

1. SUBJECT OF THE PROTOCOL AND REPORTING

1.1 Scope of the technical protocol

This Technical Protocol applies to all varieties of ***Hordeum vulgare* L.**

The protocol describes the technical procedures to be followed in order to meet the requirements of Council Regulation 2100/94 on Community Plant Variety Rights. The technical procedures have been agreed by the Administrative Council and are based on documents agreed by the International Union for the Protection of New Varieties of Plants (UPOV), such as the General Introduction to DUS (UPOV Document TG/1/3 http://www.upov.int/en/publications/intro_dus.htm), its associated TGP documents
(http://www.upov.int/en/publications/tgp/) and the relevant UPOV Test Guideline TG/19/11 dated 20th September 2018 http://www.upov.int/edocs/tgdocs/en/tg019.pdf for the conduct of tests for Distinctness, Uniformity and Stability.

1.2 Entry into Force

The present protocol enters into force on 01.08.2019. Any on-going DUS examination of candidate varieties started before the aforesaid date will not be affected by the approval of the Technical Protocol. Technical examinations of candidate varieties are carried out according to the TP in force when the DUS test starts. The starting date of a DUS examination is considered to be the due date for submitting of plant material for the first test period.

In cases where the Office requests to take-over a DUS report for which the technical examination has either been finalized or which is in the process to be carried out at the moment of this request, such report can only be accepted if the technical examination has been carried out according to the CPVO TP which was in force at the moment when the technical examination started.

1.3 Reporting between Examination Office and CPVO and Liaison with Applicant

1.3.1 Reporting between Examination Office and CPVO

The Examination Office shall deliver to the CPVO a preliminary report ("the preliminary report") no later than two weeks after the date of the request for technical examination by the CPVO.

The Examination Office shall also deliver to the CPVO a report relating to each growing period ("the interim report") and, when the Examination Office considers the results of the technical examination to be adequate to evaluate the variety or the CPVO so requests, a report relating to the examination ("the final report").

The final report shall state the opinion of the Examination Office on the distinctness, uniformity and stability of the variety. Where it considers those criteria to be satisfied, or where the CPVO so requests, a description of the variety shall be added to the report. If a report is negative the Examination Office shall set out the detailed reasons for its findings.

The interim and the final reports shall be delivered to the CPVO as soon as possible and no later than on the deadlines as laid down in the designation agreement.

1.3.2 Informing on problems in the DUS test

If problems arise during the course of the test the CPVO should be informed immediately so that the information can be passed on to the applicant. Subject to prior permanent agreement, the applicant may be directly informed at the same time as the CPVO particularly if a visit to the trial is advisable.

1.3.3 Sample keeping in case of problems

If the technical examination has resulted in a negative report, the CPVO shall inform the Examination Office as soon as possible in case that a representative sample of any relevant testing material shall be kept.

2. MATERIAL REQUIRED

2.1 Plant material requirements

Information with respect to the agreed closing dates and submission requirements of plant material for the technical examination of varieties can be found on http://www.cpvo.europa.eu/main/en/home/documents-and-publications/s2-gazette in the special issue S2 of the Official Gazette of the Office. General requirements on submission of samples are also to be found following the same link.

2.2 Informing the applicant of plant material requirements

The CPVO informs the applicant that

- He is responsible for ensuring compliance with any customs and plant health requirements.
- The plant material supplied should be visibly healthy, not lacking in vigour, nor affected by any important pest or disease.
- The plant material should not have undergone any treatment which would affect the expression of the characteristics of the variety, unless the competent authorities allow or request such treatment. If it has been treated, full details of the treatment must be given.

2.3 Informing about problems on the submission of material

The Examination Office shall report to the CPVO immediately in cases where the test material of the candidate variety has not arrived in time or in cases where the material submitted does not fulfil the conditions laid down in the request for material issued by the CPVO.

In cases where the examination office encounters difficulties to obtain plant material of reference varieties the CPVO should be informed.

3. METHOD OF EXAMINATION

3.1 Number of growing cycles

The minimum duration of tests should normally be two independent growing cycles.

3.2 Testing Place

Tests are normally conducted at one place. In the case of tests conducted at more than one place, guidance is provided in TGP/9 "Examining Distinctness"
http://www.upov.int/export/sites/upov/en/publications/tgp/documents/tgp_9_1.pdf.

3.3 Conditions for Conducting the Examination

The tests should be carried out under conditions ensuring satisfactory growth for the expression of the relevant characteristics of the variety and for the conduct of the examination.

The optimum stage of development for the assessment of each characteristic is indicated by a number in the third column of the Table of Characteristics. The stages of development denoted by each number are described in Chapter 8.2.

3.4 Test design

Each test should be designed to result in a total of at least 2000 plants, which should be divided between at least two replicates.

The assessment of the characteristic "seasonal type" should be carried out on at least 300 plants.

If ear rows are conducted, at least 100 ear rows should be observed.

In case of hybrids, the parent lines have to be included in the test and should be tested and assessed as any other self-pollinating variety. The observations on the hybrid variety itself should be made on at least 200 plants.

The design of the tests should be such that plants or parts of plants may be removed for measurement or counting without prejudice to the observations which must be made up to the end of the growing cycle.

3.5 Additional tests

In accordance with Article 83(3) of Council Regulation No. 2100/94 an applicant may claim either in the Technical Questionnaire or during the test that a candidate has a characteristic which would be helpful in establishing distinctness. If such a claim is made and is supported by reliable technical data, an additional test may be undertaken providing that a technically acceptable test procedure can be devised.

Additional tests will be undertaken, with the agreement of the President of CPVO, where distinctness is unlikely to be shown using the characters listed in the protocol.

3.6 Constitution and maintenance of a variety collection

The process for the constitution and the maintenance of a variety collection can be summarized as follows:

Step 1: Making an inventory of the varieties of common knowledge

Step 2: Establishing a collection ("variety collection") of varieties of common knowledge which are relevant for the examination of distinctness of candidate varieties

Step 3: Selecting the varieties from the variety collection which need to be included in the growing trial or other tests for the examination of distinctness of a particular candidate variety.

3.6.1 Forms of variety collection
The variety collection shall comprise variety descriptions and living plant material, thus a living reference collection. The variety description shall be produced by the EO unless special cooperation exists between EOs and the CPVO. The descriptive and pictorial information produced by the EO shall be held and maintained in a form of a database

3.6.2 Living Plant Material
The EO shall collect and maintain living plant material of varieties of the species concerned in the variety collection.

3.6.3 Range of the variety collection
The living variety collection shall cover at least those varieties that are suitable to climatic conditions of a respective EO.

3.6.4 Making an inventory of varieties of common knowledge for inclusion in the variety collection
The inventory shall take into account the list of protected varieties and the official, or other, registers of varieties, in particular:
The inventory shall include varieties protected under National PBR (UPOV contracting parties) and Community PBR, varieties registered in the Common Catalogue, the OECD list, the Conservation variety list and varieties in trade or in commercial registers for those species not covered by a National or the Common Catalogue.

3.6.5 Maintenance and renewal/update of a living variety collection
The EO shall maintain seeds in conditions which will ensure germination and viability, periodical checks, and renewal as required. For the renewal of existing living material the identity of replacement living plant material shall be verified by conducting side-by-side plot comparisons between the material in the collection and the new material.

4. ASSESSMENT OF DISTINCTNESS, UNIFORMITY AND STABILITY

The prescribed procedure is to assess distinctness, uniformity and stability in a growing trial.

4.1 Distinctness

4.1.1 General recommendations
It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 9 'Examining Distinctness' (http://www.upov.int/export/sites/upov/en/publications/tgp/documents/tgp_9_1.pdf) prior to making decisions regarding distinctness. However, the following points are provided for elaboration or emphasis in this Technical Protocol.

To assess distinctness of hybrids, a pre-screening system on the basis of the parental lines and the formula may be established according to the following recommendations:

(i) description of parental lines according to the Technical Protocols;

(ii) check of the distinctness of the parental lines in comparison with the reference collection, based on the characteristics in the table of characteristics in order to screen the closest inbred lines;

(iii) check of the distinctness of the hybrid formula in comparison with those of the hybrids in common knowledge, taking into account the closest inbred lines;

(iv) assessment of the distinctness at the hybrid level of varieties with a similar formula.

Further guidance is provided in document TGP/8 "Trial Design and Techniques Used in the Examination of Distinctness, Uniformity and Stability".

4.1.2 Consistent differences

The differences observed between varieties may be so clear that more than one growing cycle is not necessary. In addition, in some circumstances, the influence of the environment is not such that more than a single growing cycle is required to provide assurance that the differences observed between varieties are sufficiently consistent. One means of ensuring that a difference in a characteristic, observed in a growing trial, is sufficiently consistent is to examine the characteristic in at least two independent growing cycles.

4.1.3 Clear differences

Determining whether a difference between two varieties is clear depends on many factors, and should consider, in particular, the type of expression of the characteristic being examined, i.e. whether it is expressed in a qualitative, quantitative, or pseudo-qualitative manner. Therefore, it is important that users of these Technical Protocols are familiar with the recommendations contained in the UPOV-General Introduction to DUS prior to making decisions regarding distinctness.

If distinctness is assessed using the t-test least significant difference the difference between two varieties is clear if it occurs with the same sign at the 1% significance level or less ($p<0.01$) in two consecutive or two out of three growing cycles.

If distinctness is assessed by the combined over years distinctness analysis (COYD) the difference between two varieties is clear if the respective characteristics are different at the 1% significance level or less ($p<0.01$) in a test over either two or three years.

If the significance level or statistical methods proposed are not appropriate the method used should be clearly described.

4.1.4 Number of plants/parts of plants to be examined

Unless otherwise indicated, for the purposes of distinctness, all observations on single plants should be made on 10 plants or parts taken from each of 10 plants and any other observations made on all plants in the test, disregarding any off-type plants.

In the case of observations of parts taken from single plants, the number of parts to be taken from each of the plants should be 1.

4.1.5 Method of observation

The recommended method of observing the characteristic for the purposes of distinctness is indicated by the following key in the third column of the Table of Characteristics (see document TGP/9 "Examining Distinctness", Section 4 "Observation of characteristics"):

MG: single measurement of a group of plants or parts of plants
MS: measurement of a number of individual plants or parts of plants
VG: visual assessment by a single observation of a group of plants or parts of plants
VS: visual assessment by observation of individual plants or parts of plants

Type of observation: visual (V) or measurement (M)
"Visual" observation (V) is an observation made on the basis of the expert's judgment. For the purposes of this document, "visual" observation refers to the sensory observations of the experts and, therefore, also includes smell, taste and touch. Visual observation includes observations where the expert uses reference points (e.g. diagrams, example varieties, side-by-side comparison) or non-linear charts (e.g. colour charts). Measurement (M) is an objective observation against a calibrated, linear scale e.g. using a ruler, weighing scales, colorimeter, dates, counts, etc.

Type of record: for a group of plants (G) or for single, individual plants (S)
For the purposes of distinctness, observations may be recorded as a single record for a group of plants or parts of plants (G), or may be recorded as records for a number of single, individual plants or parts of plants (S). In most cases, "G" provides a single record per variety and it is not possible or necessary to apply statistical methods in a plant-by-plant analysis for the assessment of distinctness.

In cases where more than one method of observing the characteristic is indicated in the Table of Characteristics (e.g. VG/MG), guidance on selecting an appropriate method is provided in document TGP/9, Section 4.2.

With respect to the use of protein electrophoresis, the Office follows the actual UPOV approach as laid down under point 10 of this protocol. If electrophoresis is used for testing distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied. However, a sequential analysis approach could be applied to reduce the workload.

Electrophoretic characteristics with a lack of uniformity shall not be taken into account for the assessment of distinctness.

4.2 Uniformity

4.2.1 It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 10 'Examining Uniformity' (http://www.upov.int/export/sites/upov/en/publications/tgp/documents/tgp_10_1.pdf) prior to making decisions regarding uniformity. However, the following points are provided for elaboration or emphasis in this Technical Protocol:

4.2.2 The assessment of uniformity for hybrid varieties depends on the type of hybrid and should be according to the recommendations for hybrid varieties in the General Introduction.

4.2.3 In addition to the examination of the uniformity of the hybrid variety itself, uniformity of the parent lines should also be assessed.

Uniformity is assessed by visual observation and the detection of off-types.

4.2.4 The recommended sample size for the assessment of uniformity is indicated by the following key in the table of characteristics:

A: sample size of 100 plants/parts of plants/ear rows
B: sample size of 2000 plants or, in case of hybrids, 200 plants

4.2.5 For the assessment of uniformity in a sample size of 2000 plants, the following standards should be applied.

For self-pollinated varieties a population standard of 0.1 % and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 2000 plants, 5 off-types are allowed.

For male sterile lines, a population standard of 0.2 % and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 2000 plants, 8 off-types are allowed.

For male sterile single cross hybrids used as parent in a 3-way-hybrid, a population standard of 0.5% and an acceptance probability of at least 95% should be applied. In case of a sample size of 2000 plants, 15 off-types are allowed.

4.2.6 For the assessment of uniformity in a sample of 100 ear-rows, plants or parts of plants, a population standard of 1 % and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 100 ear-rows, plants or parts of plants, 3 off-types are allowed. An ear-row is considered to be an off-type ear-row if there is more than 1 off-type plant within that ear-row.

4.2.7 For "A" characteristics, with the exception of characteristic 1, the assessment of uniformity can be done in 2 steps. In a first step, 20 plants or parts of plants are observed. If no off-types are observed, the variety is declared to be uniform. If more than 3 off-types are observed, the variety is declared not to be uniform. If 1 to 3 off-types are observed, an additional sample of 80 plants or parts of plants must be observed.

4.2.8 For the assessment of uniformity of hybrid varieties, a population standard of 10% and an acceptance probability of at least 95% should be applied. In case of characteristics indicated by B, the sample size for the assessment of uniformity may be reduced to 200 plants. In case of a sample size of 200 plants, 27 off-types are allowed. In case of a sample size of 100 ear rows, plants or parts of plants, 15 off-types are allowed.

4.2.9 For all varieties except hybrid varieties, a re-submission of plant material may be allowed for the second growing cycle if in the first growing cycle the number of off-types did not exceed 15 plants in a sample size of 2000 plants (Population standard of 0.5% with an acceptance probability of ≥ 95%) or 9 plants, parts of plants or ear rows in a sample size of 100 (Population standard of 5% with an acceptance probability of ≥ 95%).

4.3 Stability

4.3.1 It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 11 'Examining Stability' (http://www.upov.int/export/sites/upov/en/publications/tgp/documents/tgp_11_1.pdf)
In practice, it is not usual to perform tests of stability that produce results as certain as those of the testing of distinctness and uniformity. However, experience has demonstrated that, for many types of variety, when a variety has been shown to be uniform, it can also be considered to be stable.

4.3.2 Where appropriate, or in cases of doubt, stability may be further examined by testing a new seed stock to ensure that it exhibits the same characteristics as those shown by the initial material supplied.

5. GROUPING OF VARIETIES AND ORGANIZATION OF THE GROWING TRIAL

5.1 The selection of varieties of common knowledge to be grown in the trial with the candidate varieties and the way in which these varieties are divided into groups to facilitate the assessment of distinctness are aided by the use of grouping characteristics.

5.2 Grouping characteristics are those in which the documented states of expression, even where produced at different locations, can be used, either individually or in combination with other such characteristics: (a) to select varieties of common knowledge that can be excluded from the growing trial used for examination of distinctness; and (b) to organize the growing trial so that similar varieties are grouped together.

5.3 The following have been agreed as useful grouping characteristics:

(a) Lower leaves: hairiness of leaf sheaths (characteristic 4)

(b) Ear: number of rows (characteristic 14)

(c) Ear: development of sterile spikelets (characteristic 15)

(d) Grain: rachilla hair type (characteristic 24)

(e) Grain: hairiness of ventral furrow (characteristic 27)

(f) Seasonal type (characteristic 29)

5.4 If other characteristics than those from the TP are used for the selection of varieties to be included into the growing trial, the EO shall inform the CPVO and seek the prior consent of the CPVO before using these characteristics.

6. INTRODUCTION TO THE TABLE OF CHARACTERISTICS

6.1 Characteristics to be used

The characteristics to be used in DUS tests and preparation of descriptions shall be those referred to in the table of characteristics. All the characteristics shall be used, providing that observation of a characteristic is not rendered impossible by the expression of any other characteristic, or the expression of a characteristic is prevented by the environmental conditions under which the test is conducted or by specific legislation on plant health. In the latter case, the CPVO should be informed.
The Administrative Council empowers the President, in accordance with Article 23 of Commission Regulation N°874/2009, to insert additional characteristics and their expressions in respect of a variety.

States of expression and corresponding notes
In the case of qualitative and pseudo-qualitative characteristics, all relevant states of expression are presented in the characteristic. However, in the case of quantitative characteristics with 5 or more states, an abbreviated scale may be used to minimize the size of the Table of Characteristics. For example, in the case of a quantitative characteristic with 9 states, the presentation of states of expression in the Test Guidelines may be abbreviated as follows:

State	Note
small	3
medium	5
large	7

However, it should be noted that all of the following 9 states of expression exist to describe varieties and should be used as appropriate:

State	Note
very small	1
very small to small	2
small	3
small to medium	4
medium	5
medium to large	6
large	7
large to very large	8
very large	9

6.2 Example Varieties

Where appropriate, example varieties are provided to clarify the states of expression of each characteristic.

6.3 Legend

For the CPVO N° column:

G	Grouping characteristic	– see Chapter 5
QL	Qualitative characteristic	
QN	Quantitative characteristic	
PQ	Pseudo-qualitative characteristic	
(+)	See Explanations on the Table of Characteristics in Chapter 8.1	

For the UPOV N° column:

The numbering of the characteristics is provided as a reference to the UPOV guideline.

(*)	UPOV Asterisked characteristic – Characteristics that are important for the international harmonization of variety descriptions.

For the column "stage, method":

00 – 99	See Explanations on the Table of Characteristics in Chapter 8.2	
MG, MS, VG, VS	Method of observation	– see Chapter 4.1.5
A	Sample size 100	– see Chapter 4.2
B	Sample size 2000 (200 for hybrids)	– see Chapter 4.2

7. TABLE OF CHARACTERISTICS

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
1.	**1.**	**00**	**Kernel: colour of aleurone layer**			
(+)		**VG/A**	whitish	Grace	California	1
			light grey blue	Henley	SY Leoo	2
PQ			dark grey blue	-	Saffron	3
			purple	-	-	4
			black	-	-	5
2.	**2.**	**25-29**	**Plant: growth habit**			
(+)	**(*)**	**VG/B**	erect	-	-	1
QN			semi-erect	Pirona	-	3
			intermediate	Grace	California	5
			semi prostrate	Quench	KWS Joy	7
			prostrate	-	-	9
3.	**3.**	**25-29**	**Plant: Intensity of green colour**			
QN		**VG/B**	light	-	Lomerit	1
			medium	Conchita	Henriette	2
			dark	Quench	KWS Meridian	3
4.	**4.**	**25-29**	**Lowest leaves: hairiness of leaf sheath**			
QL	**(*)**	**VG/A**	absent	Grace	California	1
G			present	-	Henriette	9
5.	**5.**	**45-49**	**Flag leaf: anthocyanin colouration of auricles**			
QN	**(*)**	**VG/B**	absent or very weak	-	California	1
			weak	Pirona	-	3
			medium	Conchita	SY Leoo	5
			strong	Grace	Semper	7
			very strong	-	Meseta	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
6.	**6.**	**49-51**	**Flag leaf: attitude**			
(+)		**VG/B**	erect	-	Hobbit	1
QN			semi-erect	Natasia	California	3
			horizontal	Quench	Saffron	5
			semi-reflexed	Arcadia	Matros	7
			reflexed	-	Augusta	9
7.	**7.**		**Time of ear emergence**			
(+)	**(*)**	**MG/B**	early	Lilly	Meseta	3
QN			medium	Natasia	California	5
			late	-	Saffron	7
8.	**8.**	**50-60**	**Flag leaf: glaucosity of sheath**			
QN		**VG/B**	absent or very weak	-	-	1
			weak	-	-	3
			medium	Pirona	Saffron	5
			strong	Grace	California	7
			very strong	-	Henriette	9
9.	**9.**	**60-65**	**Awns: anthocyanin coloration of tips**			
QN	**(*)**	**VG/B**	absent or very weak	-	California	1
			weak	Pirona	Lomerit	3
			medium	Ebson	Marielle	5
			strong	Grace	Semper	7
			very strong	Willma	-	9
10.	**10.**	**65-75**	**Ear: glaucosity**			
QN	**(*)**	**VG/B**	absent or very weak	Sunshine	Henriette	1
			weak	Michelle	Matros	3
			medium	Arcadia	Semper	5
			strong	Natasia	KWS Meridian	7

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
11.	**11.**	**70-80**	**Ear: attitude**			
(+)		**VG/B**	erect	-	-	1
QN			semi-erect	Quench	KWS Meridian	3
			horizontal	Grace	Saffron	5
			semi-drooping	Ingmar	Augusta	7
			drooping	-	-	9
12.	**12.**	**80-85**	**Grain: anthocyanin coloration of nerves of lemma**			
QN		**VG/B**	absent or very weak	-	California	1
			weak	Chamonix	Hobbit	3
			medium	Quench	Marielle	5
			strong	Grace	Atenon	7
			very strong	-	Matros	9
13.	**13.**	**80-92**	**Plant: length**			
(+)	**(*)**	**MS/B; VG/B**	short	Frontier	Findora	3
QN			medium	Quench	Henriette	5
			long	Pirona	Semper	7
14.	**14.**	**80-92**	**Ear: number of rows**			
QL	**(*)**	**VG/B**	two	Grace	California	1
G			six	Olsok	Henriette	2
15.	**15.**	**80-92**	**Ear: development of sterile spikelets**			
(+)	**(*)**	**VG/B**	non or rudimentary	Grace	California	1
QL G			full	Quench	Casanova	2
16.	**16.**	**80-92**	**Sterile spikelet: attitude**			
(+)	**(*)**	**VG/A**	parallel	Pirona	California	1
QN			parallel to divergent	Henley	KWS Joy	2
			divergent	Quench	Casanova	3

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
17.	**17.**	**80-92**	**Ear: shape**			
(+)	**(*)**	**VG/B**	strongly tapering	KWS Irina	California	1
PQ			slightly tapering	Arcadia	Hobbit	2
			parallel	Natasia	Semper	3
			fusiform	-	-	4
18.	**18.**	**80-92**	**Ear: density**			
(+)	**(*)**	**MS/B; VG/A**	sparse	Ingmar	Casanova	3
QN			medium	Quench	KW Meridian	5
			dense	Belgravia	Findora	7
			very dense	Mercada	-	9
19.	**19.**	**80-92**	**Ear: length**			
(+)		**MS/B; VG/B**	short	Mercada	KWS Meridian	3
QN			medium	Quench	Findora	5
			long	Ingmar	California	7
20.	**20.**	**80-92**	**Awn: length**			
(+)	**(*)**	**MS/B VG/B**	very short	Pirona	-	1
			short	Marthe	KWS Meridian	3
QN			medium	Natasia	Augusta	5
			long	Quench	Lomerit	7
			very long	-	-	9
21.	**21.**	**92**	**Rachis: length of first segment**			
QN		**MG/A**	short	Quench	SY Leoo	3
		MS/A	medium	Natasia	KS Meridian	5
		VG/A	long	Belgravia	California	7

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
22.	**22.**	**92**	**Rachis: curvature of first segment**			
(+)		**VG/A**	absent or very weak	-	-	1
QN			weak	KWS Aliciana	Henriette	3
			medium	Henley	California	5
			strong	Ingmar	KWS Joy	7
23.	**23.**	**92**	**Median spikelet: length of glume and its awn relative to grain**			
(+)	**(*)**	**VG/A**	shorter	-	-	1
QN			equal	Quench	California	2
			slightly longer	-	Cierzo	3
			much longer	-	KWS Meridian	4
24.	**24.**	**80-92**	**Grain: rachilla hair type**			
(+)	**(*)**	**VG/A**	short	Quench	KWS Joy	1
QL G			long	Grace	California	2
25.	**25.**	**80-92**	**Grain: spiculation of inner lateral nerves of dorsal side of lemma**			
(+)		**VG/A**	absent or very weak	Grace	California	1
QN			weak	Chamonix	KWS Joy	3
			medium	Henley	SY Leoo	5
			strong	-	Semper	7
26.	**26.**	**92**	**Grain: type**			
QL	**(*)**	**VG/A**	non-husked	Pirona	-	1
			husked	Grace	Henriette	9
27.	**27.**	**92**	**Grain: hairiness of ventral furrow**			
(+)	**(*)**	**VG/A**	absent	Grace	Henriette	1
QL G			present	-	Saffron	9
28	**28**		**Lemma: shape of base**			
(+)		**VG/A**	non-bevelled		Montana	1
QL			bevelled	Grace	Henriette	2

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
29.	**29.**		**Seasonal type**			
(+)		**VG**	winter type	-	Henriette	1
PQ			alternative type	-	Farandole	2
G			spring type	Grace	Cierzo, Genie	3

8. EXPLANATIONS ON THE TABLE OF CHARACTERISTICS

8.1 Explanations for individual characteristics

Ad 1: Kernel: colour of aleurone layer

The colour of the aleurone layer should be assessed visually after the kernel is put in water over night. If necessary, a magnifying glass may be used.

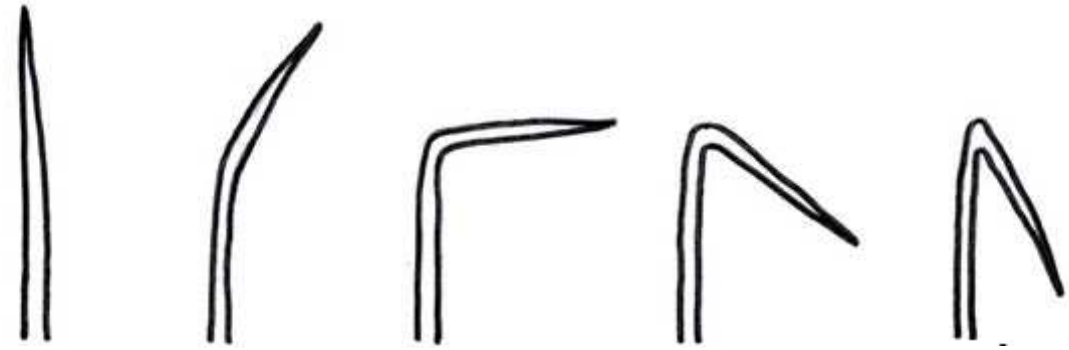
Ad 2: Plant: growth habit

The growth habit should be assessed visually from the attitude of the leaves and tillers. The angle formed by the outer leaves and the tillers with an imaginary vertical axis should be used.

1	3	5	7	9
erect	semi-erect	intermediate	semi-prostrate	prostrate

Ad 6: Flag leaf: attitude

Flag leaf attitude is sensitive to the stage of plant development. Therefore, observation at the appropriate stage (BBCH 49 – 51) is of particular importance.
Flag leaf attitude relates to the angle between the main axis (stem) and the flag leaf blade. The expression of the majority of plants should be recorded without considering individual plants which may express a different attitude.

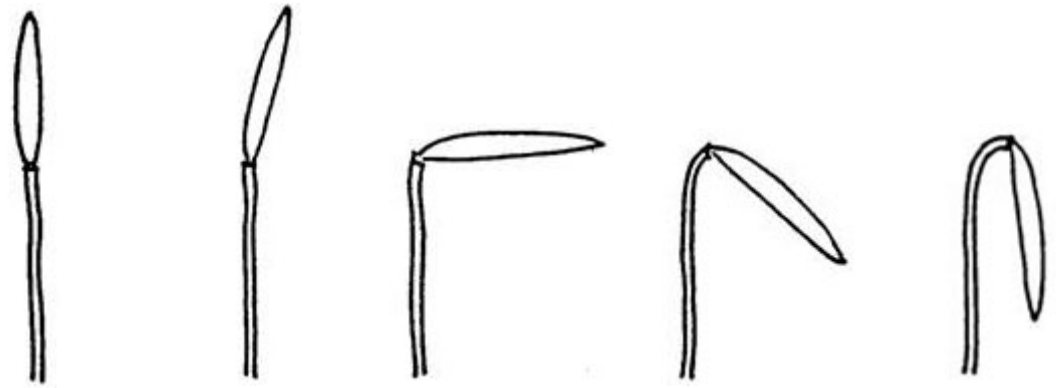


1	2	3	4	5
erect	semi-erect	horizontal	semi-drooping	drooping

Ad 7: Time of ear emergence

Time of ear emergence is reached when the first spikelet is visible on 50% of ears.

Ad 11: Ear: attitude



1 erect | 3 semi-erect | 5 horizontal | 7 semi-drooping | 9 drooping

Ad. 13: Plant: length

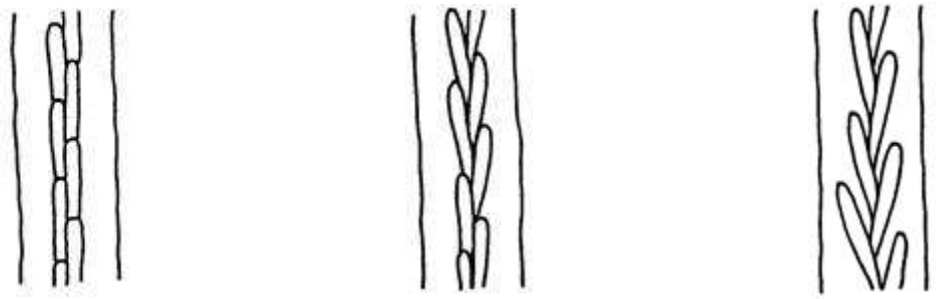
Plant length includes stem, ear and awns.

Ad. 15: Ear: development of sterile spikelets

Observation of sterile spikelet is only applicable for two-row varieties.

Ad. 16: Sterile spikelet: attitude

The attitude of sterile spikelets should only be observed for varieties with fully developed spikelets. Observations should be done in the middle third of the ear.



1 parallel | 2 parallel to divergent | 3 divergent

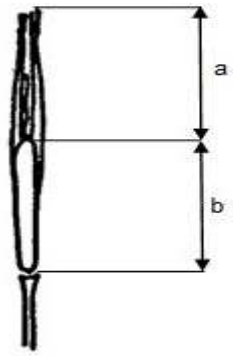
Ad 17: Ear: shape

1 strongly tapering | 2 slightly tapering | 3 parallel | 4 fusiform

Ad. 18: Ear: density

The density is the ratio of the number of spikelets per ear length.

Ad 19: Ear: length



a = awn length
b = ear length

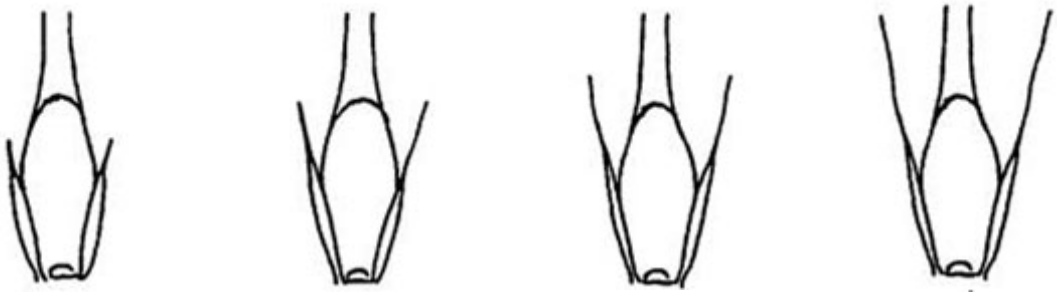
Ad 20: Awn: length

See Ad. 19

Ad 22: Rachis: curvature of first segment

1	3	5	7	9
absent or very weak	weak	medium	strong	very strong

Ad 23: Median spikelet: length of glume and its awn relative to grain



1	2	3	4
shorter	equal	slightly longer	much longer

Ad 24: Grain: rachilla hair type

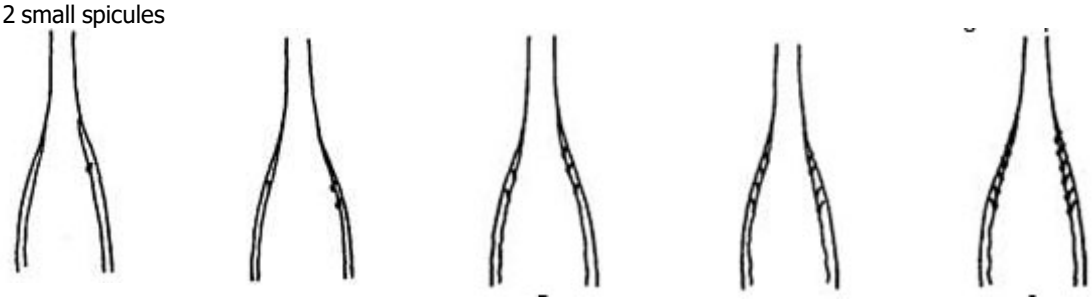
1
short

2
long

Ad 25: Grain: spiculation of inner lateral nerves of dorsal side at lemma

None or occasionally 1 or 2 small spicules

10 or more large regular spicules



1
absent or very weak

3
weak

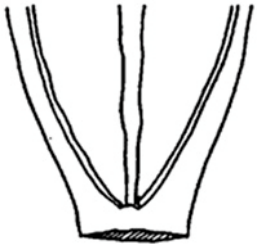
5
medium

7
strong

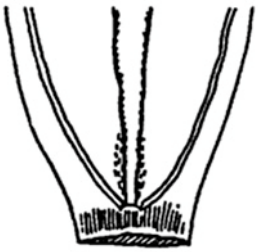
9
very strong

Ad 27: Grain: hairiness of ventral furrow

The ventral furrow should be observed after moving the rachilla. It is of particular importance to have installed the light source at the right place. A very little number of hairs should be assessed as "present".



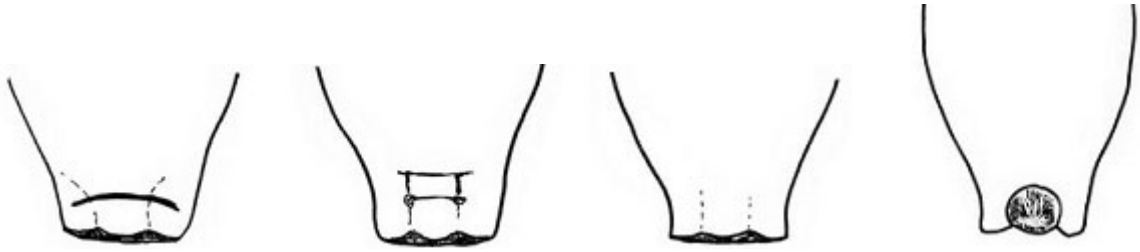
1
absent



9
present

Ad. 28: Lemma: shape of base

Observations should be made in the middle third of the ear. In the case of six row varieties, observations should be made in the middle row of spikelets.



1
non-bevelled

2
bevelled

Ad. 29: Seasonal type

The seasonal type (need of vernalisation) should be assessed on plots sown in springtime. Example varieties should always be included in the trial. When the example varieties behave according to their descriptions, the varieties under study can be described. At the time when the latest spring type variety is fully mature (stage 91-92 of the Zadoks decimal code) the growth stage reached by the respective variety should be assessed. The states of expression are defined as follows:

1 - Winter type (high need of vernalisation): The plants have reached stage 45 of the Zadoks decimal code (boots swollen) at maximum.

2 - Alternative type (partial need of vernalisation): The plants have exceeded stage 45 of the Zadoks decimal code (they should normally have exceeded stage 75) and have reached stage 90 at maximum.

3 - Spring type (no need or very weak need of vernalisation): The plants have exceeded stage 90 of the Zadoks decimal code.

Seasonal type is not related to winter hardiness. Spring type varieties have no need for vernalisation but may have winter hardiness.

8.2 Growth stages

The descriptions of the growth stages of the Zadoks decimal code for cereals (ZADOKS et al., 1974)

Zadoks Decimal code	Description
	Germination
00	Dry seed
01	Start of imbibition
03	Imbibition complete
05	Radicle emerged from seed
07	Coleoptile emerged from seed
09	Leaf just at coleoptile tip
	Seedling growth
10	First leaf through coleoptile
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
14	4 leaves unfolded
15	5 leaves unfolded
16	6 leaves unfolded
17	7 leaves unfolded
18	8 leaves unfolded
19	9 or more leaves unfolded
	Tillering
20	Main shoot only
21	Main shoot and 1 tiller
22	Main shoot and 2 tillers
23	Main shoot and 3 tillers
24	Main shoot and 4 tillers
25	Main shoot and 5 tillers
26	Main shoot and 6 tillers
27	Main shoot and 7 tillers
28	Main shoot and 8 tillers
29	Main shoot and 9 or more tillers
	Stem elongation
30	Pseudo stem erection
31	1st node detectable
32	2nd node detectable
33	3rd node detectable
34	4th node detectable
35	5th node detectable
36	6th node detectable
37	Flag leaf just visible
39	Flag leaf ligule/collar just visible
	Booting
41	Flag leaf sheath extending
43	Boots just visibly swollen
45	Boots swollen
47	Flag leaf sheath opening
49	First awns visible
	Inflorescence emergence
50	First spikelet of inflorescence visible
51	-
53	1/4 of inflorescence emerged
55	1/2 of inflorescence emerged
57	3/4 of inflorescence emerged
59	Emergence of inflorescence completed
	Anthesis
60	Beginning on anthesis
65	Anthesis half-way
69	Anthesis completed
	Milk development
71	Caryopses watery ripe
73	Early milk
75	Medium milk
77	Late milk
	Dough development
80	-
83	Early dough
85	Soft dough
87	Hard dough
	Ripening
91	Caryopses hard (difficult to divide with thumbnail)
92	Caryopses hard (can no longer be dented with thumbnail)
93	Caryopses loosening in daytime
94	Overripe, straw dead and collapsing
95	Seed dormant
96	Viable seed giving 50% germination
97	Seed not dormant
98	Secondary dormancy induced
99	Secondary dormancy lost

9. LITERATURE

Zadok, J.C., Chang, T.T., Konzak, C.F., 1974: A Decimal code for the Growth Stages of Cereals. Weed Research. NL, 14: 415-421

10. ELECTROPHORESIS

10.1 Introduction

The following Annex contains a list of characteristics based on storage proteins revealed by electrophoresis and a description of the method to be used. UPOV decided to place these characteristics in an Annex to the Test Guidelines, thereby creating a special category of characteristic, because the majority of the UPOV members is of the view that it is not possible to establish distinctness solely on the basis of a difference found in a characteristic based on storage proteins revealed by electrophoresis. Such characteristics should therefore only be used as a complement to other differences in morphological or physiological characteristics. UPOV reconfirms that these characteristics are considered useful but that they might not be sufficient on their own to establish distinctness. They should not be used as a routine characteristic but at the request or with the agreement of the applicant of the candidate variety.

For the analysis of hordeins, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE) is recommended. Hordeins are encoded by three compound loci known as Hor-1, Hor-2 and Hor-3 located on chromosome 5 (Hor-1 and Hor-2 on the short arm, Hor-3 on the long arm). There are a number of alleles at each locus and the analysis of hordeins is based on the recognition of these alleles from proteins, which appear on gels as a series of well-defined bands or patterns of bands. The loci encode different groups of electrophoretically separable proteins, known as B-, C- and D-hordeins in decreasing order of mobility. The alleles at each locus can be designated by letters or numbers, or a combination of both. The relative electrophoretic mobilities (REMs) of each of the bands can also be determined.

If only C-(Hor-1) and B-(Hor-2) hordeins are of interest, then the standard reference acid PAGE method of the International Seed Testing Association (ISTA) could be used.

10.2 Characteristics derived by Protein Polymorphism

	Characteristics Band position in SDS PAGE method	Band position in Acid PAGE method	Example Varieties	Note
30.	**D-Hordein composition: allele expression at locus Hor-3**			
	band 34		(W) California	1
	band 33		(W) Medina	2
	band 35		(W) Saturn	3
	band 32.5		(W) Iris	4
	band 32		(W) Princesse	5
31.	**C-Hordein composition: allele expression at locus Hor-1**			
	bands 62+65+68	bands 27+30+32+37+39	(W) California	1
	bands 62+65+66+68	bands 27+30+32+34+37+39	(W) Lomerit	2
	bands 65+68	bands 27+30+32+37	(W) Medina	3
	bands 66.5+71	bands 32+37+41	(W) Sandra	4
	bands 61.5+66.5+71	bands 27+30+32+37+39+41	(S) Meltan	5
	bands 65	bands 32+37+38	(S) Armada	6
	bands 60 +67.5+68.5	bands 35+38	(W) Roseval	7
	bands 61+65+68+73	bands 32+37+39+41	(W) Semper	8
	bands 60+69+72	bands 38+41+42	(S) Sydney	9
	bands 64+66.5	bands 30+32+37	(W) Saturn	10
	bands 67+71	bands 34+37	(S) Pastello	11
	bands 65+68+69+70	bands 34+39+41+42	(W) Albacete	12
	bands 61.5+68+71	bands 31+34+37+38+41	(W) Borwina	13
	bands 65+67.5	bands 32+37+41+43	(W) Kendo	14
	bands 65.5+70.5		(W) Delita	15
	bands 66+70.5		(W) Maybrit	16

	Characteristics		Example Varieties	Note
	Band position in SDS PAGE method	Band position in Acid PAGE method		
32.	**B-Hordein composition: allele expression at locus Hor-2**			
	bands 79+86+88+100	bands 71+79+83+86+94+100	(S) Quench	1
	bands 79+88+91+95+97+101	bands 71+82+89+100	(S) Overture	2
	bands 79+91+92+95+97+101	bands 76+82+83+86+100	(S) Hellana	3
	bands 75+82+87+91+97	bands 66+71+76+86+93+100	(W) Caribic	4
	bands 79+86+88+97+101	bands 71+78+79+90+94	(W) Piroline	5
	bands 78+84+95+101	bands 76+81+94	(W) Ingmar	6
	bands 79+90+91+94+100	bands 71+72+75+82+85+86+100	(S) Sebastian	7
	bands 78+86+91+95+100	bands 72+76+79+90+94	(W) Sandra	8
	bands 79+82+88+91+92+100	bands 71+76+79+86	(S) Ebson	9
	bands 76+79+86+88+100	bands 71+78+83+86+94+100	(S) Trebon	10
	bands 79+86+89+92+95+101	bands 71+79+83+86+90	(W) Sigma	11
	bands 79+95+101	bands 71+76+79	(W) Midas	12
	bands 78+89+92+101	bands 71+89	(W) Lomerit	13
	bands 75+78+79+81+89+101	bands 79+83+86+90	(W) Findora	14
	Bands 75+78+79+81+83+86+88+94+95+100	bands 67+69+71+72+78+79+85+89+94	(W) Caresse	15
	bands 81+84+88+90+101	bands 71+79+83+88+94	(W) Reseda	16
	bands 75+78+79+81+83+86	bands 69+76+79+83+93	(W) Baronesse	17
	bands 82+88+100	bands 71+72+79+85+86+91+100	(W) Albacete	18
	bands 81+100	bands 72+76+100	(S) Basic	19
	bands 75+79+83+89+91	bands 61+71+76+79+83	(W) Camargue	20
	bands 79+84+92	bands 76+81+94+100	---	21
	bands 79+91+92		(W) Libelle	22
	bands 75+79+91+92+95+97+101		(W) Anja	23
	bands 75+79+90+94+99		(W) Hiberna	24
	bands 79+(83-85)+(89-91)+(94-96) +102		(W) Jerka	25

10.3 Description of the method to be used

1. SDS PAGE Method for Analysis of Hordeins from *Hordeum vulgare*

1.1 Apparatus and equipment

Any suitable vertical electrophoresis system can be used, provided that the gels can be kept at a constant temperature. A gel thickness of no more than 1.5 mm is recommended. The power supply used should be capable of delivering both constant current and constant voltage output.

1.2. Chemicals

All chemicals should be of 'Analytical Reagent' grade or better.

Acrylamide (specially purified for electrophoresis)
Bisacrylamide (specially purified for electrophoresis)
Tris (hydroxymethyl) methylamine (TRIS)
Sodium dodecyl sulphate (SDS)
Ammonium persulphate (APS)
2-mercaptoethanol
TEMED (NNN'N'-tetramethylethylenediamine)
Trichloroacetic acid (TCA)
Hydrochloric acid
Glacial acetic acid
Glycine
n-Butanol
Pyronin
Glycerol (d = 1.256)
Methanol
Coomassie Brilliant Blue R-250 (or equivalent)
Coomassie Brilliant Blue G-250 (or equivalent)

1.3 Solutions

1.3.1 Extraction solution

Stock solution:
6.25 ml 1M TRIS HCl buffer, PH 6.8 (see 1.3.3.2)
12.05 ml distilled water
2g SDS
10 mg Pyronin
10 ml glycerol
This solution can be stored for 2 months at 4°C.

Immediately before use; extraction solution is prepared as follows:

28.33 ml stock buffer solution plus 7.91 ml 2-mercaptoethanol made up to 100 ml with distilled water. This solution must be prepared immediately prior to use and cannot be stored.

1.3.2 Electrophoresis (running) buffer

Stock solution:
141.1 g glycine
30.0 g TRIS
10.0 g SDS
made up to 1 liter with distilled water.

Immediately before use, the stock solution is diluted 1:10 with distilled water.

The stock buffer solution can be stored for 2 months at room temperature. Do not store the diluted buffer more than one week. The pH of the buffer must be close to 8.3.

1.3.3 Gel preparation solutions

1.3.3.1 Stock resolving gel buffer (1M TRIS HCl pH 8.8)

121.14 g TRIS plus approximately 20 ml HCl (d = 1.19) made up to 1 liter with distilled water. This buffer can be stored at 4°C for 2 months.

1.3.3.2 Stock stacking gel buffer (1M TRIS HCl, pH 6.8)

121.14 g TRIS plus approximately 78 ml HC1 (d = 1.19) made up to 1 liter with distilled water. This buffer can be stored at 4°C for 2 months.

1.3.3.3 10% (w/v) SDS solution

10g of SDS dissolved in distilled water and made up to 100 ml. This solution can be stored at 4°C for 2 months. Prior to use, stir and heat gently to re-dissolve the SDS, if it comes out of solution.

1.3.3.4 1% (w/v) ammonium persulphate solution

1 g of APS dissolved in distilled water and made up to 10 ml. This solution must be prepared immediately prior to use.

1.3.3.5 Stock acrylamide solution

51.98 g acrylamide made up to 100 ml with distilled water.

1.3.3.6 Stock bisacrylamide solution

0.3185g bisacrylamide made up to 130 ml with distilled water.

1.3.4 Staining solutions

1.3.4.1 0.25g Coomassie Brilliant Blue G-250 plus 0.75g Coomassie Brilliant Blue R-250, made up to 100 ml with water.

1.3.4.2 55 g TCA, 65 ml glacial acetic acid, 180 ml methanol plus 25 ml solution 1.3.4.1, made up to 1 liter with distilled water.

1.4 Procedure

1.4.1 Protein extraction

Individual seeds are ground using a hammer (or other device). Ground seed meal is mixed with diluted sample extraction buffer (1.3.1) in a 3 ml polypropylene hemolyse or similar tube with a screw-on cap. The ratio of meal/extraction buffer is 50 mg/0.75 ml. The samples are extracted for 2 hours at room temperature, mixed several times using a vortex mixer, heated in a boiling water bath for 10 minutes and then allowed to cool. The tubes are centrifuged at 18,000 g for 5 minutes.

According to the gel thickness and the size of the wells, the volume of extract loaded can vary. Between 10 and 25 µl is usually sufficient.

1.4.2 Preparation of the gel

Clean and dry gel cassettes are assembled, according to the design of the equipment used. If tape is used to seal the cassettes, it is advisable to assemble them at least one day in advance of use, to enable the tape to 'age' and adhere better.

1.4.2.1 Resolving (main) gel (10% acrylamide, pH 8.8)

To make two slab gels of 180 x 160 x 1.5 mm, the following is required:
20 ml stock acrylamide solution (1.3.3.5)
26 ml stock bisacrylamide solution (1.3.3.6)
30 ml stock gel buffer (1.3.3.1).

These should be at 4°C. The mixture is de-gassed in a 100 ml Buchner flask for 10 minutes. To this is added:

2 ml APS (1.3.3.4),
0.8 ml SDS (1.3.3.3),
40 µl TEMED (use straight from bottle).

The gels are then carefully poured, avoiding the formation of air bubbles, and polymerisation is allowed to take place at room temperature.

The gel cassettes should not be filled entirely, in order to leave room for a 3-4 cm layer of stacking gel. The gel surface is carefully overlaid with n-butanol (or distilled water) using a syringe. When polymerisation is finished (about 30 min), the gel surface is carefully rinsed with distilled water and dried with filter paper.

1.4.2.2 Stacking gel (3.5% acrylamide, pH 6.8)

In a 50 ml Buchner flask, mix:
1.35 ml stock acrylamide solution (1.3.3.5),
3.17 ml stock bisacrylamide solution (1.3.3.6)
2.50 ml stock gel buffer (1.3.3.2) and
12.30 ml distilled water.

Following de-gassing add:
0.875 ml APS (1.3.3.4),
0.233 ml SDS (1.3.3.3),
17.5 µl TEMED (straight from bottle)

Mix carefully and immediately pour the stacking gels to the top of the gel cassettes. Insert the well-forming "comb", avoiding air bubbles. Allow to polymerise for about 2 hours. The "combs" are then removed carefully from the gel cassettes and the wells rinsed using diluted electrophoresis running buffer (1.3.2).

1.4.3 Electrophoresis

The tank is filled with the appropriate volume of running buffer (1.3.2), cooled to 15 °C. Following sample loading, electrophoresis is carried out at a constant current of 8 mA/sq cm (cross-sectional area) of gel until the pyronin G has moved through the stacking gel, and then at 16 mA/sq cm of gel (maximum voltage 300V) until the marker is at the bottom of the gel. The temperature should be maintained at 15 °C.

1.4.4 Fixing and staining

The gel cassettes are removed from the tank, opened and the gels fixed in 250 ml of 15% (w/v) TCA for at least 30 minutes. The gels are rinsed in distilled water and stained overnight in 250 ml of staining solution (1.3.4.2) at room temperature. Distaining is not usually necessary but gels should be washed in distilled water before being stored in sealed polythene bags.

Other staining procedures can be successfully used (e.g. Coomassie Brilliant Blue G or equivalent in TCA alone). The final quality control criterion, both for gel preparation and gel staining, is to analyse the suggested example varieties on each batch of gels. The separation of the suggested bands, and their relative electrophoretic mobilities (molecular weights) must be clear in order for the procedures to be judged satisfactory.

1.5 Recognition of Hordein Alleles (SDS PAGE)

The band pattern presented in the tables for B-, C- and D-hordeins are schematic and differences in band intensity have been ignored in the presentation.

B-, C- and D-hordeins: nomenclature of the individual bands and recognition of the corresponding alleles (SDS-PAGE)

Characteristic 30: **D-Hordein composition: allele expression at locus Hor-3**

Band	Example	Note				
	California	1	2	3	4	5
32						--
32.5					--	
33			--			
34	--	--				
35				--		

Characteristic 31: C-Hordein composition: allele expression at locus Hor-1

Band	Example California	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Band
60								--										60
61									--									61
61.5						--								--				61.5
62	--	--	--															62
64											--							64
65	--	--	--	--			--		--				--		--			65
65.5																--		65.5
66			--														--	66
66.5					--	--					--							66.5
67												--						67
67.5								--							--			67.5
68	--	--	--	--					--				--	--				68
68.5								--										68.5
69										--			--					69
70													--					70
70.5																--	--	70.5
71					--	--						--		--				71
72										--								72
73									--									73

Characteristic 32: B-Hordein composition: allele expression at locus Hor-2

Band	Example Quench	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	Band
75					--										--	--		--			--			--	--		75
76											--																76
78							--		--					--	--	--		--									78
79	--	--	--	--		--		--		--	--	--	--		--	--		--			--	--	--	--	--	--	79
81															--	--	--	--		--							81
82					--														--								82
83																--		--			--					--	83
84							--										--					--				--	84
85																										--	85
86	--	--				--			--		--	--				--		--									86
87					--																						87
88	--	--	--			--				--	--					--	--		--								88
89												--		--	--						--					--	89
90								--									--								--	--	90
91			--	--	--			--	--	--											--		--	--		--	91
92				--						--		--		--								--	--	--			92
94								--								--									--	--	94
95			--	--			--		--			--	--			--								--		--	95
96																										--	96
97			--	--	--	--																		--			97
99																									--		99
100	--	--						--	--		--					--			--	--							100
101			--	--		--	--			--		--	--	--	--		--							--			101
102																										--	102

2. Acid PAGE Method for Analysis of B- and C-Hordeins from *Hordeum vulgare*

If only B- and C-hordeins are of interest, then acid PAGE can be used. The following method is the standard reference method recommended by the International Seed Testing Association.

2.1. Apparatus and Equipment

Various designs of vertical electrophoresis equipment have been used successfully, including those available from Biometra, Bio-Rad, Desaga and Pharmacia-LKB. The power supply used should be capable of operating at constant voltage and constant current.

2.2. Chemicals

All chemicals should be of "Analytical Reagent" grade or better.

Acrylamide ("specially purified for electrophoresis")
Bisacrylamide ("specially purified for electrophoresis")
Urea
Glacial acetic acid
Glycine
Ferrous sulphate
Ascorbic acid
Hydrogen peroxide
Monothioglycerol
Pyronin G
Trichloroacetic acid (TCA)
Methanol
2-chloroethanol
Coomassie Brilliant Blue G-250 (or equivalent)
Coomassie Brilliant Blue R-250 (or equivalent)

2.3 Solutions

2.3.1 Extraction solution

Pyronin G (0.05%) (w/v) in 2-chloroethanol (20%) (v/v) containing urea (18% w/v) and monothioglycerol (1% v/v) (keep cold or prepare fresh).

2.3.2 Tank buffer solution

Glacial acetic acid (4 ml) and glycine (0.4g), made up to 1 litre with distilled water, keep cold.

2.3.3 Gel buffer solution

Glacial acetic acid (20 ml) and glycine (1.0g), made up to 1 litre with distilled water, keep cold.

2.3.4 Staining solutions

0.25g Coomasie Brilliant Blue G-250 + 0.75g Coomassie Brilliant Blue R-250 in 100 ml water.

55g TCA, 65 ml glacial acetic acid, 180 ml methanol, plus 25 ml solution 2.3.4.1, made up to 1 litre with distilled water.

2.4. Procedure

2.4.1 Protein extraction

Single seeds are crushed with pliers or by similar means and transferred to 1.5 ml polypropylene centrifuge tubes or to micro-titer plates. Extraction solution (2.3.1) (0.3 ml) is added and the tubes or plates are allowed to stand overnight at room temperature. If necessary, the tubes are centrifuged at 18,000xg and the supernatants used for electrophoresis.

2.4.2 Preparation of the gel

Clean and dry gel cassettes are assembled, according to the design of the equipment. Treating the glass plates with silicon prior to assembly can facilitate subsequent removal of the gel. The gel cassettes can incorporate a plastic backing sheet (e.g. "Gel Bond PAG", FMC Corporation). This supports the gel during subsequent operations. To make 100 ml of gel medium, gel buffer at 4°C (2.3.3) (approximately 60 ml) is taken and the following added: acrylamide (10g), bisacrylamide (0.4g), urea (6g), ascorbic acid (0.1g), ferrous sulphate (0.005g). The solution is stirred and made up to 100 ml with cold (4°C) stock gel buffer solution (2.3.3). Freshly prepared 0.6% (v/v) hydrogen peroxide solution (0.35 ml per 100 ml of gel medium) is added, mixed quickly and the gel poured. An acrylic "comb" is placed in the top of the cassette, to make wells in the gel. Polymerisation is carried out at room temperature and should be complete in five to 15 minutes. If not, it may be necessary to adjust the volume of hydrogen peroxide added. The gel mixture should over-fill the cassette, or be over-layed with water, to ensure satisfactory polymerisation of the upper surface.

2.4.3 Electrophoresis

The acrylic comb is removed from the gel and the sample wells washed with tank buffer (2.3.2). The tank is filled with an appropriate volume of buffer (2.3.2) (depending on the equipment used). Samples (10-20 micro l) are loaded into the wells and the gel placed in the tank, ensuring that the sample wells are completely filled. The temperature of the lower buffer chamber should be kept at 15°C. Electrophoresis is carried out at a constant voltage of not more than 60V/cm^2 (cross-sectional area) of gel (which corresponds to a voltage of 500V for two gels 16 cm wide and 0.15 cm thick) for twice the time taken for the pyronin G marker to leave the gel. It must be remembered that the anode (positive electrode) is at the origin (top of the gel) in this system.

2.4.4 Fixing and staining

The gel cassette is removed from the tank, opened and the gel placed in a plastic box containing 200 ml of staining solution (2.3.4.2). Staining is carried out overnight at room temperature. Destaining if necessary is carried out by placing gels in water for about two to 3 hours at room temperature. Gels can then be dried or stored in sealed polythene bags at 4°C.

It should be noted that other procedures, such as the use of increased temperatures or the use of mixtures of TCA and Coomassie Brilliant Blue G, will give satisfactory staining of gels. The final quality control criterion, both for gel preparation and gel staining, is to analyse the suggested example varieties on each batch of gels. The separation of the designated bands, and their relative electrophoretic mobilities, must be clear and correct in order for the procedures to be satisfactory.

2.5 Recognition of Hordein Alleles (Acid PAGE)

B- and C-Hordeins: nomenclature of the individual bands and recognition of the corresponding alleles: acid PAGE

Characteristic 31: C-Hordein composition: allele expression at locus Hor-1

Band	Example California	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Band
25																25
27	--	--	--	--		--										27
30	--	--	--	--		--					--					30
31														--		31
32	--	--	--	--	--	--	--		--		--				--	32
34			--									--	--	--		34
35								--								35
37	--	--	--	--	--	--	--		--		--	--		--	--	37
38							--	--		--				--		38
39	--	--	--			--			--				--			39
41					--	--			--	--			--	--	--	41
42										--			--			42
43															--	43
		Alleles according to acid PAGE nomenclature														
		10	10A	1	11	17	6	19	2	4	5	18	14	8	3	

Characteristic 31: B-Hordein composition: allele expression at locus Hor-2

Band	Example Quench	Note 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Band
61																					--		61
66					--																		66
67																--							67
69																--		--					69
71	--	--	--		--	--		--		--	--	--	--	--		--	--		--		--		71
72								--	--							--			--	--			72
75								--															75
76				--	--		--		--	--			--					--		--	--	--	76
78						--					--					--							78
79	--	--				--			--	--		--	--		--	--	--	--	--		--		79
81							--															--	81
82			--	--				--															82
83	--	--									--	--			--		--	--			--		83
85								--								--			--				85
86	--	--		--	--			--		--	--	--			--				--				86
88																	--						88
89			--											--		--							89
90						--			--			--			--								90
91																			--				91
93					--													--					93
94	--	--				--	--		--		--					--	--					--	94
97																							97
100	--	--	--	--	--			--			--								--	--		--	100
		3	4	13	14	-	9	1	7	6	-	-	11	16	-	18	-	19	8	15	12	10	

11. TECHNICAL QUESTIONNAIRE

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO/TQ-019/5