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UPOV

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INTERNATIONAL UNION FOR THE PROTECTION OF NEW VARIETIES OF PLANTS

GENEVA

TECHNICAL COMMITTEE

Fifteenth Session
Geneva, March 18 and 19, 1980

IMPLICATIONS OF SOPHISTICATED METHODS SUCH AS ELECTROPHORESIS OR BIOCHEMICAL METHODS ON THE TESTING OF DISTINCTNESS

Document prepared by the Office of the Union

1. During its fourteenth session (November 1979), the Technical Committee discussed the question whether electrophoresis could be used as a method for the testing of varieties on distinctness in the procedure for granting plant breeders' rights. It was agreed to discuss this problem further during the fifteenth session of the Technical Committee. (See document TC/XIV/5, paragraph 26).

2. Two working papers have been prepared to facilitate the envisaged discussions in the Technical Committee, one by experts of the United Kingdom, the other by experts of the Netherlands. Both working papers are attached to this document as Annex I (United Kingdom) and Annex II (Netherlands).

[Two Annexes follow]

TC/XV/2

ANNEX I [Original]

ELECTROPHORESIS AS A TOOL IN DISTINCTNESS TESTING

(Working paper prepared by experts from the United Kingdom)

1. BACKGROUND

Electrophoresis as a technique has existed for a number of years. The technique is based on the fact that certain macromolecules will move through a support medium at differing rates when an electrical potential is applied across the medium. The speed of movement is dependent apart from other things, on the size of the charge on the molecule, and on the pore size of the medium. Improvements and refinements have led to varying forms of electrophoresis, such as zonal electrophoresis in various media, concentration gradient gel and isoelectric focusing and the molecules that have been subjected to this separation method range from amino acids to enzymes and proteins.

The number of different techniques that can be applied for separation is approximately a function of the following variables:-

"Molecular weight x Molecular charge x Pore medium x Buffer system x Electrical potential across medium x staining technique x recording technique."

From this it will be seen that electrophoresis is not a single technique but has many and varied possibilities.

Briefly the results of an electrophoretic separation are seen as a series of bands of different widths after appropriate staining of the support medium. These bands may differ between cultivars in their positinn, number and intensity. "Clearly distinct" would most likely be taken as pertaining to differences related to the presence or absence of bands rather than differences of intensity of similar bands.

2. CURRENT USE

Various electrophoretic techniques have been used by millers, plant breeders and research workers for a number of years. Development of specific tests is being carried out in the UK under Agricultural Research Council at Rothamsted (barley), the Plant Breeding Institute (wheat) and Welsh Plant Breeding Station (grasses). The Brewing Industry Research Foundation and the Lord Rank Research Centre are also known to be using one or more techniques. In commerce their main use appears to be as a rapid means of confirming the identity of, for example, batches of grain or flour by reference to the gliadin fraction of wheat. Other techniques have been used for the separation of isoenzymes and hordeins. Electrophoresis for DUS purposes is used in France where one technique, for the separation of the gliadin fraction of winter wheat, is used under rigorously standardised procedures. Its use is restricted to National List applications of satisfactory VCU status. It is understood that the technique is considered to be insufficiently reliable for PBR purposes for which VCU considerations are not relevant and where decisions rest solely on DUS criteria.

In Sweden plant breeders rights have been granted to two new varieties of Festuca rubra on the basis of distinguishing them electrophoretically.

3. CONSISTENCY OF RESULTS

While it is accepted that the chemistry of the technique is well established it is understood that the difficulties experienced in France referred to above have, at least in part, been caused by different batches of starch and of chemicals giving somewhat different results. In attempting to overcome this difficulty a very precise procedure has been agreed between the French testing authorities, seed certification agencies, the breeders, the millers and any others using the test. It is understood that, despite this, difficulties remain in interpreting the results to the levels of precision probably needed.

4. A ROUTINE TOOL OR A SPECIAL TEST?

Were a particular technique to be established as a routine test all breeders would need to be consulted.

The introduction of a routine test would mean that all breeders would have to conduct their own tests on their submitted material and it is doubtful whether many are in a position to do this, at least at present. Alternatively, the technique might be of use where an applicant claims a difference in his TQ submitted at the time of application. With certain limitations as to the practicability and repeatability of a test, current procedures allow for this and UPOV guidelines make reference to the fact that the lists of characteristics are not exhaustive and that others may be added when they have been found useful.

5. UNIFORMITY AND STABILITY

For the establishment of the current levels of uniformity to the current levels of confidence in the autogamous species such as wheat, barley and oats a sample of about 400 grains would have to be tested. This may present physical and workload problems. However, probably the most important problem would be for breeders in ensuring that their submitted material was sufficiently uniform in its electrophoretic reaction. Ellis (1977) reports that a number of currently listed varieties are not genetically pure for electrophoretic reaction. A further problem arises in establishing the distinctness of a new variety in an autogamous species (which should be uniform) from an existing variety (which may not be uniform) wherein a difference could be established in only a proportion of the grains examined.

Careful consideration will have to be given to the levels of uniformity which might be expected to be achieved in allogamous species.

While there is no reason to believe that a morphologically uniform cereal variety would be unstable in its electrophoretic reaction it is considered doubtful whether breeders of allogamous species such as grasses would be able

to maintain the pattern of distribution established when the variety was first listed.

6. ADVANTAGES AND DISADVANTAGES

6.1 Advantages

6.1.1. Relatively cheap labour costs, as the tests could be conducted by trained staff after the establishment of the routine procedure.

6.1.2. Relatively rapid results. For example, an electrophoretic run can be completed within 48 hours.

6.1.3. Applicable to single or part grains (but these are normally destroyed).

6.1.4. Uses little laboratory space.

6.1.5. Equipment is relatively cheap.

6.1.6. Results are not normally affected by the environment in which the plant is grown.

6.2 Disadvantages

6.2.1. Low loading can lead to anomalous results, therefore band presence rather than band intensity would be preferable.

6.2.2. Use of the technique would involve a lengthy run-in period with full consultation with the breeding organisations in order to comply with undertakings already given about the introduction of new techniques.

6.2.3. Wide application could require excessive numbers of tests if the presently accepted levels of uniformity for DUS testing (1%) and certification (0.05%) were adhered to. Any alternative would mean a reduction in the level of uniformity expected.

6.2.4. The use of whole or part seeds may preclude the use of further progeny testing for confirmation purposes.

6.2.5. Although hardly a disadvantage it should be noted that to date nearly all electrophoresis techniques have been applied to varieties which have already satisfied the existing DUS procedure. UK and French results show that varieties which are distinct morphologically, may have identical band patterns.

6.2.6. The difficulty of selecting from the wide range of electrophoretic tests available the one most suitable for the current problem.

6.2.7. Some chemicals required are subject to use restrictions imposed under Health and Safety at Work legislation.

7. COSTS

While the initial cost of laboratory equipment may be high, the cost of using electrophoretic techniques as special tests would not be large. If the tests were to be introduced as a routine test for all varieties the cost would be considerable. A possibility exists that the techniques might be so comprehensive as to replace field tests. This is mere speculation at present and is considered to be most unlikely. Thus the costs of any electrophoretic tests could be anticipated to be additional to the costs of field tests.

It is understood that at least in the initial stages an experienced and trained biochemist would be needed.

8. CONCLUSIONS

In view of the known difficulties more research into the possible application of these techniques specifically for DUS purposes must be undertaken before they could be considered for acceptance as either routine or special tests. While the potential of the techniques appears to be considerable, there is a danger of being drawn into an open-ended commitment to a wide range of tests unless a preparatory study is made.

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[Annex II follows]

TC/XV/2

ANNEX II [Original]

CONSIDERATIONS ABOUT POSSIBLE USE OF NEW TECHNIQUES, ESPECIALLY ELECTROPHORESIS, IN VARIETY RESEARCH

(Working paper prepared by experts from the Netherlands)

Introduction:

During its last session the Technical committee decided to discuss in its next meeting in March 1980 the implications on plant breeders rights arising from the introduction of sophisticated techniques.

- Protein electrophoresis serves as an example for the problem.

Other possible examples are the use or extended use of

- Gas-chromatography and high pressure liquid chromatography.
- Reactions to chemicals, including pesticides and enzyme indicators.
- Immune reactions.
- Colour analysis with visible, ultra-violet, infra-red and other light sources.
- High power microscopy.

The rather wide use of protein electrophoresis permits evaluation of the situation. Since the views taken by the authorities in the UPOV member states might be - or sometimes are - divergent a mutual consideration of the problem seems desirable.

The Technical Committee is not asked to reopen the discussions with respect to characteristics already included in the established Guidelines for the conduct of tests for distinctness, homogeneity and stability.

It is also not intended to discuss the use of modern equipment that is coming available for a more efficient or more objective assessment of established characteristics.

The discussion should, on the contrary, focus on the possible introduction as a "caractère de nouveauté" of a characteristic

a. that has not yet been agreed for the crop concerned,
b. that is not readily visible but necessitates use of specialized apparatus and skill, and
c. that, as for as is known, does not relate to the functional properties of the variety.

The Committee should evaluate with great care under what conditions introduction of characteristics is in accordance with the Preamble of the Convention, viz. when used for the granting of Plant Breeders Rights serves the purposes of the development of agriculture and the safeguarding of the interests of the breeder.

Electrophoresis

Electrophoresis denotes a chemico-physical technique in which charged particles are separated by differential movement in an electric field. When a positive and a negative electrode are brought in a fluid which contains charged particles the negative particles will move to the positive electrode (anode) and the positive particles to the negative one (kathode).

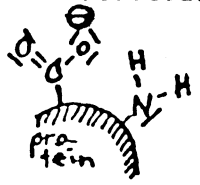
The velocity of the particles is a.o. dependent of:

1. The strength of the electric field (voltage).
2. Temperature and viscosity of the fluid.
3. The charge of the particles.
4. The dimensions and the shape of the particles.

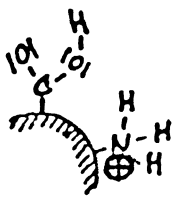
The factors 3 and 4 are properties of the particles which can be very different for different substances and consequently they can be separated due to their different velocity in an electric field.

Proteins like storage proteins and enzymes, present in juices of plant materials, are very big molecules with an electric charge.

The amount of electric charge and the polarity depend on the acidity (pH) of the solvent in the following manner: In an acid environment the alkaline structures of a molecule (e.g. $-NH_2$) are dissociated and have an electric charge while the acid structures have no charge.



alkaline environment

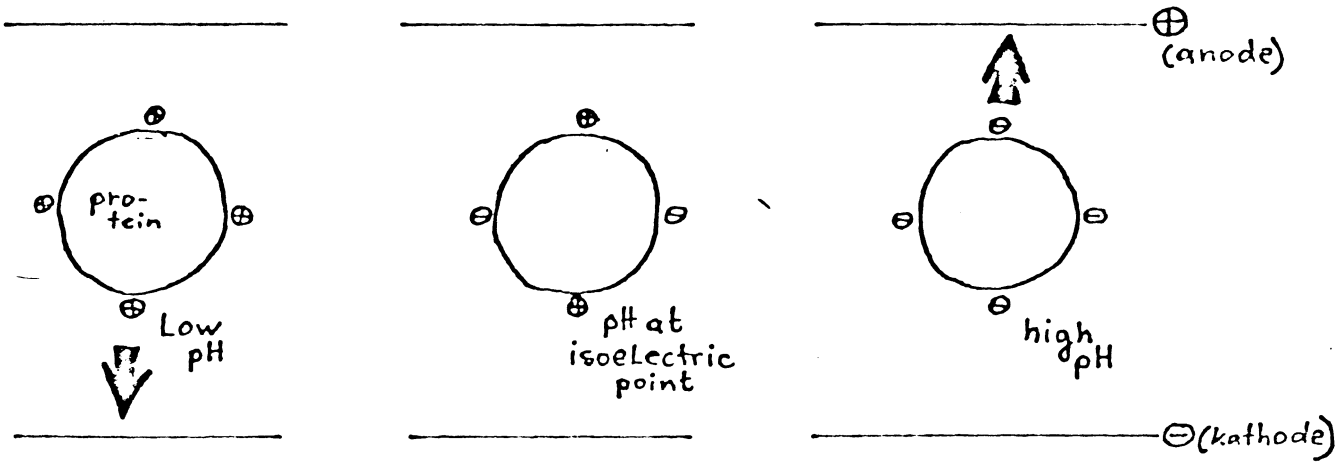


acidic environment

In an alkaline environment the opposite is true (see figure). The direction of movement of the protein molecules therefore is not only dependent on the polarity of the electric current but also of the acidity (pH) of the solvent.

This also means that for every kind of protein molecules there is a special acidity of the solvent at which the same amount of acidic and alkaline groups on a molecule are dissociated which makes the molecule as a whole electrially neutral and not moving if a current is applied.

This acidity (pH) has a different value for the different kinds of proteins and is called the isoelectric point.



more positive than negative charges. The protein moves to the kathode.

the same amount of positive and negative charges. The protein does not move.

more negative than positive charges. The protein moves to the anode.

The isoelectric point is used in a special kind of electrophoresis which is called the "isoelectric focusing".

In principle electrophoresis can be carried out in different materials like paper or porous plastic films but mostly today gels of agar, starch or polyacrylamide are used. The polyacrylamide gel is a 3-dimensional net-like molecular system and is obtained by polymerisation of acrylamide and a crosslinking agent ethylenebis-acrylamide. This accounts for the name polyacrylamidegelelectrophoresis (PAGE). Polyacrylamide gel has the advantage over other carrier substances that the pore width can be regulated by the concentration of the monomer and of the crosslinking agent.

Since the charge of the protein molecules depends on the pH of the solvent electrophoresis must take place in a buffered solution. Therefore the gel is polymerised in buffer in order to keep the pH constant and at the required level during the run.

The molecules of the protein mixture to be analysed are moving in the gel due to the electric current and when they have migrated over a sufficient distance the gel is taken out of the apparatus and the protein bands are made visible by staining. This staining can be made a-specifically by means of compounds that give colour to all proteins in the gel but also specifically on certain enzyme systems. When the a-specific procedures are used (e.g. staining with Coomassie Brilliant Blue) only proteins which are present in greater quantities will become visible. The specific procedures make it possible to visualise very small quantities of enzyme. This is done by the use of a compound that is specifically converted to a colored insoluble compound by the enzymes in the gel. At all places in the gel where they are present coloured material appears resulting in a banding pattern. Staining reagents of this type are developed for many enzyme systems.
The mostly used types of electrophoresis are:

1. "Ordinary" electrophoresis: The proteins move in a gel with homogeneous pore-width and at constant pH.
2. Density-gradient electrophoresis: The proteins move in a gel of constant pH but with gradually smaller pores along the migrating path.
3. pH-gradient electrophoresis (Isoelectric focusing): The proteins move in a gel of constant pore width but in gradually changing pH. This means that a molecule at a certain place in the gel meets a pH at which it is electrically neutral and will not move further. (The isoelectric point). Every component of the protein mixture is therefore accumulating in the gel where the pH is equal to its isoelectric point. Very sharp separations are possible with this technique and banding patterns are generated consisting of a great amount of very narrow bands.
4. SDS-electrophoresis: The above procedures no. 1 and 2 can be used but the protein mixture is treated with a powerful surfactant (Sodium-Dodecyl-Sulfate) with which is achieved that the protein molecules are desintegrated into subunits.

Band patterns obtained in this way have been proven to be highly typical for the plant varieties used and independent of the environmental conditions in which the variety has been grown. For example it is known that extra nitrogen manuring will raise the protein content of the plant but the relative amounts of the different components in the mixture will not change. Also factors like climate, weather or soiltype do not affect the pattern.

Different parts of the plant give specific electropherograms.
Electrophoresis and its variants can reveal small differences, e.g. between isoenzymes = proteins catalyzing the same chemical reaction but differing in minor parts of their structure.

At present electrophoresis is applied in practice for the identification of seed lots of wheat and barley and for the identification of potatoes.
It is further used for sib-control in the production of hybrid varieties in Brassica.
There is no doubt that electrophoresis is a powerful tool for identification purposes.
Whether or not this technique is acceptable for the admittance of new varieties is a separate question.
The answer depends on the weighing of a number of considerations, a.o.

1) The number of different techniques seems to be virtually unlimited.
2) Application of a certain technique for the admittance of a variety leads to the obligation to apply this method also with respect to maintenance and control of this variety.
3) Once a method is introduced for distinctness purposes for one variety the same method and at least the same level of homogeneity has to be applied to all varieties under test from then onwards.
4) It is probable that within any existing variety subselections can be made, maintained and reproduced that differ in electrophoretic characters without being a separate variety in the present understanding of the word.

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[End of Annex II
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