RESOLUTION OIV/OENO 427/2010

CRITERIA FOR THE METHODS OF QUANTIFICATION OF POTENTIALLY ALLERGENIC RESIDUES OF FINING AGENT PROTEINS IN WINE

The GENERAL ASSEMBLY,

CONSIDERING Article 2 paragraph 2 iv of the agreement establishing the International Organisation of Vine and Wine,

Upon the proposal of the Sub-commission of Methods of Analysis and Appraisal of Wine,

CONSIDERING that the O.I.V's activities shall to help protect the health of consumers and to contribute to food safety;

Points out that these criteria should be reconsidered taking into account decisions of other international bodies,

Recognises that specific methods for the determination of potentially allergenic residues of fining agent proteins in wine are not prescribed and several ELISA methods already available can be applied.

DECIDES: to adopt the criteria below for the methods of quantification of potentially allergenic residues of fining agent proteins in wine:

CRITERIA FOR THE METHODS OF QUANTIFICATION OF POTENTIALLY ALLERGENIC RESIDUES OF FINING AGENT PROTEINS IN WINE

1. Method Criteria Definitions

- Trueness :the closeness of agreement between the average value obtained from a large series of test results and an accepted reference value
- r = Repeatability limit, the value below which the absolute difference between 2 single test results obtained under repeatability conditions (i.e., same sample, same operator, same apparatus, same laboratory, and short interval of time) may be expected to lie within a specific probability (typically 95%) and hence r = 2.8 x S_r.
- S_r = Standard deviation, calculated from results generated under repeatability conditions.

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- RSD_r= Relative standard deviation, calculated from results generated under repeatability conditions [(S_r/ $\bar{x}$) x 100], where $\bar{x}$ is the average of results over all laboratories and samples.
- R = Reproducibility limit, the value below which the absolute difference between single test results obtained under reproducibility conditions (i.e., on identical material obtained by operators in different laboratories, using the standardised test method), may be expected to lie within a certain probability (typically 95%); R = 2.8 x S_R.
- S_R = Standard deviation, calculated from results under reproducibility conditions.
- RSD_R= Relative standard deviation calculated from results generated under reproducibility conditions [(S_R/ $\bar{x}$ x 100]
- H_{OR} = HORRAT value: the observed RSDR value divided by the RSDR value calculated from the Horwitz equation.
- B_0 = Mean blank
- LOD = Limit of detection, calculated as LOD = B_0 + 3*$S_r(0)$
- LOQ = Limit of quantification, calculated as LOD = B_0 + 10*$S_r(B_0)$

2. General Aspects

Requirement

The method of analysis must be associated with specific oenological practices

Additives or processing aids containing allergenic proteins

Each product must be characterized from the chemical point of view and quality control is strictly necessary

Class of analytical methods

Generally speaking, immunoenzymatic approaches are considered the most suitable and easy methods for routine control of allergens.

The determination of allergenic fining agent proteins residues in wines could use Sandwich, Competitive, Direct or Indirect ELISA methods.

If no enzyme-labeled antibody is available a biotinylated antibody and avidine-HRP

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conjugate can be used for detection.

Antibody

- Antibody characterization (evaluation of detection of allergens with higher or lower affinity)
- High specificity for the commercial processing aids (characterized as described above)
- Cross-reactivity characterization taking in account the proteins usually included in enological practices
- Capability to detect allergen derivatives that could be formed by enological treatments (proteolysis or modified molecules)

Method

- Antibody must have optimal binding properties in wine samples
- Methods must have optimal performances in wine samples having different chemical characteristics (pH and dry extract, red and white wine, etc..)
- Results in wines coming from different geographical area (even when different enological practices are applied) must be comparabl
- The binding properties of the antibodies must be optimal with different condition of maturation of wine (time, temperatures, color changes ...)

3. Type of methods

Specific methods for the determination of fining agent proteins in wine are not prescribed yet. Several ELISA methods are already available and can be applied.

Laboratories shall use a method validated to OIV requirements that fulfils the performance criteria indicated in Table 1. Wherever possible, the validation shall include a certified reference material in the collaborative trial test materials. If not available, an alternative estimation of trueness should be used.

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The General Protocol for the Direct and Indirect ELISA Method

The direct, one-step method uses only one labeled antibody. This labeled antibody is incubated with the antigen contained in the sample/standard and bound to the well.

The indirect, two-step method uses a labeled secondary antibody for detection. First, a primary antibody is incubated with the antigen contained in the sample/standard and bound to the well. This is followed by incubation with a labeled secondary antibody that recognizes the primary antibody.

Direct

1. Prepare a surface to which antigen in sample is bound.
2. Block any non-specific binding sites on the surface.
3. Apply enzyme-linked antibodies that bind specifically to the antigen.
4. Wash the plate, so that the antibody-enzyme conjugates in excess (unbound) are removed.
5. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
6. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

Before the assay, the antibody preparations must be purified and conjugated.

Indirect

1. Prepare a surface to which antigen in sample is bound.
2. Block any non-specific binding sites on the surface.
3. Apply primary antibodies that bind specifically to the antigen
4. Wash the plate, so that primary antibodies in excess (unbound) are removed.
5. Apply enzyme-linked secondary antibodies which are specific to the primary antibodies.
6. Wash the plate, so that the antibody-enzyme conjugates in excess (unbound) are removed.

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7. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
8. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

Before the assay, both antibody preparations must be purified and one must be conjugated.

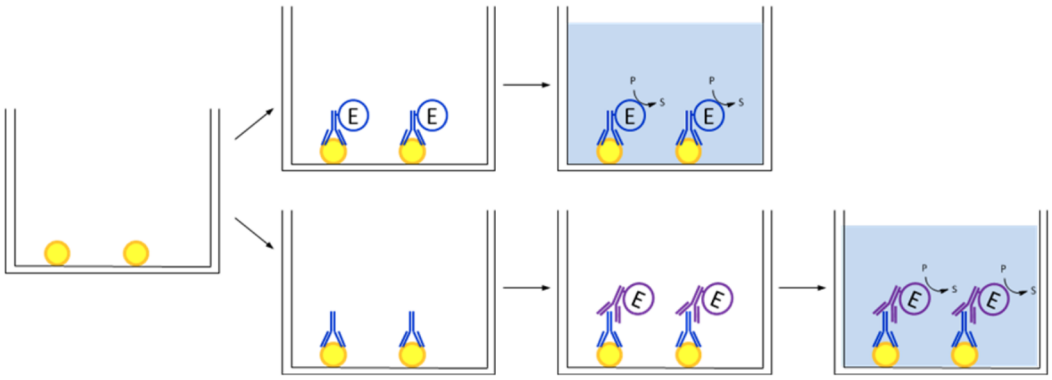


Figure 1: Direct and indirect ELISA

For most applications, a high-binding polystyrene microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for binding the given antigen.

The major advantage of direct and indirect ELISA is the high sensitivity, achieved via a comparably easy set-up with reduced chances of unspecific binding. However, it is only applicable in samples containing low amounts of non-antigen protein.

General Protocol for the competitive ELISA Method

The term "competitive" describes assays in which measurement involves the quantification of a substance by its ability to interfere with an established system. The detection can be done directly, one-step method, or indirectly, two-step method.

Direct

1. Prepare a surface to which a known quantity of wanted antigen is bound.

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2. Block any non-specific binding sites on the surface.
3. Apply the sample or standard (antigen) and the enzyme-linked antibodies that bind specifically to the antigen on the coated microplate. The antigens immobilized on the surface and the antigens in solution “compete” for the antibodies. Hence, the more antigen in the sample, the less antibody will be bound to the immobilized antigens.
4. Wash the plate so that the antibodies in excess (unbound) and unbound antigen-antibody-complexes are removed.
5. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
6. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

Before the assay, the antibody preparations must be purified and must be conjugated.

Indirect

1. Prepare a surface to which a known quantity of antigen is bound.
2. Block any non-specific binding sites on the surface.
3. Apply the sample or standard (antigen) and the specific primary antibody to the coated microplate. The antigens immobilized on the surface and the antigens in solution “compete” for the antibodies. Hence, the more antigen in the sample, the less antibody will be bound to the immobilized antigens.
4. Wash the plate so that the antibodies in excess (unbound) and unbound antigen-antibody-complexes are removed.
5. Add a secondary antibody, specific to the primary antibody, conjugated with an enzyme.
6. Wash the plate so that the conjugated antibodies in excess (unbound) are removed
7. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
8. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

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Before the assay, both antibody preparations must be purified and one must be conjugated.

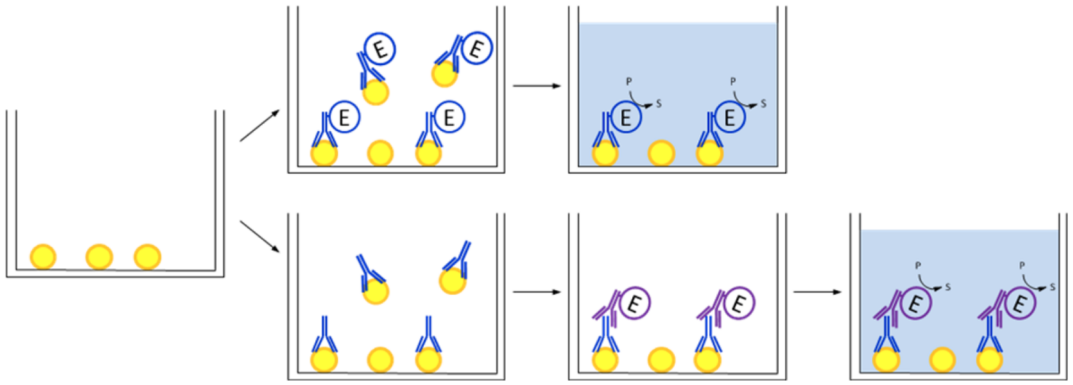


Figure 2: Direct and indirect competitive ELISA

For competitive ELISA, the higher the original antigen concentration, the weaker is the signal.

For most applications, a high-binding polystyrene microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for binding the given antigen.

General Protocol for the Sandwich ELISA Method

The Sandwich ELISA measures the amount of antigen between two layers of antibodies (i.e. capture and detection antibody). The antigen to be measured must contain at least two different antigenic sites (epitopes) for binding two different antibodies. Either monoclonal or polyclonal antibodies can be used.

Direct

1. Prepare a surface to which capture antibody is bound.
2. Block any non-specific binding sites on the surface.
3. Apply the antigen-containing sample or standard to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. Apply enzyme-linked antibodies (detection antibodies) that bind specifically to the

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antigen.

6. Wash the plate, so that the enzyme-linked antibodies in excess (unbound) are removed.
7. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
8. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

Before the assay, both antibody preparations must be purified and one must be conjugated.

Indirect

1. Prepare a surface to which capture antibody is bound.
2. Block any non specific binding sites on the surface.
3. Apply the antigen-containing sample or standard to the plate.
4. Wash the plate, so that unbound antigen is removed.
5. Apply primary antibodies that bind specifically to the antigen.
6. Wash the plate, so that primary antibody in excess (unbound) is removed.
7. Apply enzyme-linked antibodies (secondary antibodies) that bind specifically to the primary antibody.
8. Wash the plate, so that the enzyme-linked antibodies in excess (unbound) are removed.
9. Apply a chemical which is converted by the enzyme into a color or fluorescent or electrochemical signal.
10. Measure the absorbance or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen.

Before the assay, all the antibody preparations must be purified and one of them must be conjugated.

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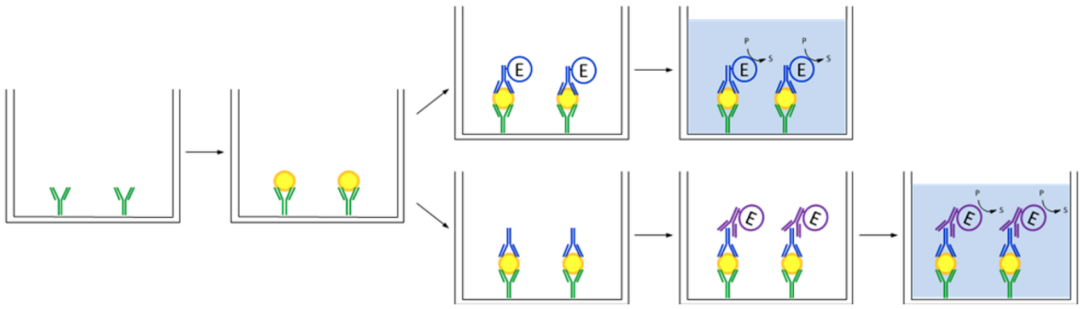


Figure 3: Direct and indirect Sandwich-ELISA

For indirect Sandwich-ELISA, it is necessary for the capture antibodies and the detection antibodies to be raised in different species (e.g. mouse and rabbit), so that the enzyme-linked secondary antibodies specific for the detection antibodies do not bind to the capture antibodies, as well.

For most applications, a high-binding polystyrene microtiter plate is best; however, consult manufacturer guidelines to determine the most appropriate type of plate for binding the given antigen.

For sandwich ELISA, the measure is proportional to the amount of antigen in samples.

The advantage of Sandwich ELISA is that even crude samples do not have to be purified before analysis, and the assay can be very sensitive.

Table 1: Performance criteria for methods of analyses for potentially allergenic fining agent proteins in wine**Parameter**	**Value/Comment**
Applicability	Suitable for determining fining agents in wine for official purposes.
Detection limit	(expressed in mg/L) Casein: at least 0.5 Ovalbumin: at least 0.5 Isinglass : at least 0.5 Lysozyme : at least 0.5

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Limit of quantification	(expressed in mg/L) Casein: at least 1 mg/L Isinglass : at least 1 mg/L Lysozyme : at least 1 mg/L Ovalbumin : at least 1 mg/L
Precision	HORRAT values of less or equal to 2 in the validation collaborative trial
Recovery	80% - 105% (as indicated in the collaborative trial)
Specificity	Free from matrix interferences
Trueness	$\lvert\bar{x} - m\rvert < 1{,}96 * \sqrt{S_{R(lab)^2} - S_{r(lab)^2} * (1 - 1/n)}$ where m is the certified value of the wine reference material and $\bar{x}$ is the average of n measurements of compound content in this wine, within the same laboratory. $S_{r(lab)}$ are standard deviations, calculated from results within the same laboratory under repeatability conditions. $S_{R(lab)}$ are standard deviations, calculated from results within different laboratories under reproducibility conditions.

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