

TECHNICAL COMMUNICATIONS

Policy on Characterization of Antibodies Used in Immunochemical Methods of Analysis for Mycotoxins and Phycotoxins

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The purpose of this document is to provide a policy on antibody characterization for conducting AOAC collaborative studies for immunochemical methods submitted for AOAC® Official MethodsSM Program status. The policy defines recommended information and characteristics to be provided by the Study Director, in the protocol of the collaborative study, for approval by AOAC. The document specifies parameters for characterization of antibodies used as biological reagent in the protocol of validation of immunochemical methods for the determination of mycotoxins and phycotoxins. These recommendations are applicable to the validation of any method, whether proprietary or non-proprietary, that is submitted to AOAC for Official Methods of AnalysisSM status recognition.

Immunochemical methods are based on the ability of antibodies to bind specifically different substances. The reversible association between antibodies and their corresponding antigens is called the immunological reaction. The binding forces involve hydrogen bonds and hydrophobic binding as well as weak molecular interactions like Coulomb and Van der Waals forces. Antigens are characterized by their immunogenicity as well as their antigenicity, which resides in their ability to initiate the production of antibodies in animals and subsequently interact or bind with them. Small peptides and small nonpeptidic molecules, such as mycotoxins and phycotoxins, are not immunogenic and are called haptens. Once hapten is conjugated to a protein, it becomes immunogenic. Sensitive immunological methods were developed involving labelling techniques to measure the complex formation when the analyte, i.e., the phycotoxin, is present at concentrations less than $\mu\text{g-mg/mL}$. Generally, the terms “immunochemical methods” or “immunoassays” are used to refer

to such methods (1). Among different labelling techniques used in immunoassay, the best known are radioimmunoassay (RIA), enzyme immunoassay (EIA), and enzyme-linked immunosorbent assay (ELISA). Immunoabsorption methods are based on temporary immobilization of immunocomplexes on a solid support. Immunoaffinity chromatography (IAC) is a means of retaining a given antigen from a complex matrix by using the anti-antigen antibodies. In this case, antibodies are immobilized on a support and packed in a small column. This type of immunochemical method is considered more as a cleanup step of a specific analytical method than an immunoassay itself (2). However, modification of IAC can be made to achieve an analytical purpose. The overall performance of any immunoassay is a function not only of the immunological principle, but also of the properties of the reagents, the properties of the sample matrix, the assay design, and the experimental errors. These basic principles determine the sensitivity, specificity, precision, and accuracy of the assay. The general protocol for the development of immunochemical methods on mycotoxins and phycotoxins includes 4 areas: (i) preparation of immunogen: this includes the formation of a toxin derivative (if necessary) and conjugation of it to a protein; (ii) production of antibodies: this includes immunization, collection of the immune serum, and purification of antibodies; (iii) characterization of antibodies: this includes determination of antibody titer and antibody specificity; (iv) development and application of immunochemical techniques (1).

This document is focused mainly on the third area to specify parameters for antibody characterization.

Types of Antibodies

Polyclonal Antibodies

Animals can produce several different types of immunoglobulins which bind, at a specific site (epitope), the antigen or hapten. These immunoglobulins are synthesized by several cell types of lymphocytes. Such immune serum contains polyclonal antibodies which generally are not mono-specific for a the given antigen/hapten. Thus, these types of purified antibody can react or “cross-react”

Table 1. Checklist for the description of antibodies used in immunochemical methods

Parameter	Specific information required	
Type of antibody	Polyclonal (host)	Monoclonal (cell line reference number)
Antibody purification	e.g., Raw serum fraction, or purified by immunoaffinity chromatography or other procedure (purification method stated)	Purification method stated, when it has been used
Avidity	Mention the Association Constant (calculation method stated or referenced; e.g., 50% inhibition value of standard curve, if applicable, for example ELISA-type test systems)	
Specificity (cross-reactivity) of related antigens (applicable for ELISA-type test systems)	Mention ALL relevant compounds with % cross-reactivity. Substances with cross-reactivities of less than 10% may be summarized (for example "other aflatoxins tested with cross reactivities of less than 10% were aflatoxin Bx, CGx..."). The basis of calculation of cross-reactivities should be mentioned (e.g., comparison of 50% inhibition values for Analytes A, B, C...). According to the specificity pattern, indicate whether the assay is (1) quantitative, (2) semiquantitative, or (3) qualitative.	
Matrix effects (nonspecific binding inhibition) applicable for use of antibodies in ELISA-type and IAC systems	Define whether or not matrix effects (false-positive, false-negative) may occur for certain sample matrixes using the respective method protocol. Mention in the scope or applicability section in what matrixes (commodities, foods, etc.) the antibody and/or system is intended to be used.	
Selectivity (binding) of antigens in IAC-type extract purification systems	List all compounds which are specifically bound by the antibodies used in IAC under the test protocol	
Specific capacity of IAC columns	Indicate the maximum amount of antigen per column bound under working conditions. If applicable (multi-analyte columns), indicate whether or not, and to which extent, presence and binding of analyte A may interfere with binding of analyte B.	

with some structurally related antigen/hapten/analyte. The source of these antibodies stops with the death of the immunized animals (1).

Monoclonal Antibodies

It is very difficult to obtain antisera with identical properties from different animals. Even from the same animal, the antisera successively collected at different times can have different properties. These limitations of polyclonal antibodies (pAb) justify attempts to produce monoclonal antibodies (mAb) which have identical physical, biochemical, and immunological properties. The antibody-producing lymphocytes are isolated and then fused with myeloma cells. The hybrid cells are selected in a particular culture medium and then screened for their specific antibody produced. The result is a cell line clone which only produces a specific mAb. These cells can be maintained in vitro over long periods of time and can generate large quantities of identical antibody. They are theoretically immortal (1).

Recommendation

It is recommended that the type of antibodies (monoclonal or polyclonal) should be clearly mentioned in the collaborative study protocol for the immunoassay validation. It is also recommended that poly- and monoclonal antibodies have to be purified. Consequently, the purification method should be stated or reference of the method should be given. In the case of mAb, when the producing cell line was referenced, the reference clone number should be indicated.

The type of immunoglobulins (Igs) should be determined. In the case of IgG, the concentration can be measured using the following equation:

$$\text{IgG, mg/mL} = A / \epsilon$$

where A is UV absorption at 280 nm and ϵ is 1.45 for pure IgG.

Avidity-Affinity

Principle

Affinity and avidity are often used synonymously and both relate to the energy of binding of a particular antigen-antibody combination. The term avidity refers specifically to the properties of an antibody, and the term affinity to those of the antigen. Numerically, avidity of the antibody for the antigen is equal to affinity of the antigen for the antibody. The affinity or avidity of the antibody has a strong influence on the further performance of the developed immunoassay or immuno-purification chromatography and may be calculated, if the equilibrium constant is known. The antigen-antibody reaction may be described using reaction kinetics as well as thermodynamic equations. However, also time and temperature have influence on assay performance parameters.

In addition, the apparent performance characteristic of competitive immunoassay formats is dependent on the avidity for the labelled antigen, which is usually different from that of the free antigen. In IAC applications, avidity and affinity are identical due to the noncompetitive conditions. However, for

multi-analyte systems, different affinities for different antigens may interfere with each other.

Recommendation

It is recommended to include data on avidity of the antibodies, more adequately, the equilibrium constant for the antibody-hapten interaction used in the immunoassay as complementary information in the collaborative study protocol for the immunoassay validation. The method of determination (or estimation) of the avidity should be stated or reference of the method should be given.

Specificity

Principle

Next to avidity, the specificity of an antibody is paramount for the performance of the assay. In principle, a specific reaction in immunology may be defined as follows: In the presence of different molecules, the specific antibodies must complex only one kind of molecules. The probability of forming a "wrong" complex determines the specificity of the reaction. In other words, specificity is determined by the steric (tridimensional) match of antigen and antibody as well as by the number of molecular interactions taking place between both molecules. Discussion of specificity requires that both the structure of the antigen and the homogeneity or heterogeneity of the antibodies be considered. An antibody preparation is homogenic, if all the antibodies bind only the one and the same epitope, although with different affinity. This condition is fulfilled by monoclonal antibodies, but may also be by polyclonal antibodies against compounds of low molecular weight (haptens). On the other hand, an antibody preparation is heterogenic, if it contains different antibody populations specific for different epitopes.

On a molecular basis, "specific" cross-reactivity describes the case in which at least 2 different antigens compete for the same antibody binding site. Almost exclusively, true cross-reactivity is observed in competitive assays using either monoclonal or polyclonal antibodies against low molecular weight compounds. In practice, this means that a high enough concentration of a truly cross-reacting substance gives the same result as the "right" substances in a competitive assay (3).

Also, nonspecific influence on many instrumental assay can occur. Nonspecific cross-reactivity includes many factors that influence the assay including unknown compounds with similar epitopes as the target compound (e.g., matrix effects) and environmental effects (steric effects, pH, temperature, etc.). Such interference occurrence often cannot be distinguished from the specific influence of cross-reacting substances. In both cases of specific and nonspecific cross-reactivities, the assay response is the same and a false positive or a false negative result may be obtained. Due to the immunoassay principle, however, false negatives are less likely found in competitive assays providing the assay is well optimized.

Procedure

Test specificity describes the extent to which presence of substances with chemical structure or structural parts similar to that of the original analyte (test substance) in a sample will also result in competitive binding and, thus, give a positive result in the test system (cross-reaction). Test specificity is a result of antigen-antibody reaction which is determined by the immunoreagents used in the test (antibody, labelled antigen) and must, therefore, not be commingled with nonspecific influences on the test system (e.g., sample matrix interference; sample solution pH; 4).

The specificity study should be performed under the conditions which closely match the "real" analyte situation during sample analysis. To avoid the introduction of anomalous effects due, for example, to solvation effects on the analyte, the solvent concentration for the preparation of the standard curve should be identical with those of analysis of the sample.

In a competitive immunoassay such as those used in the analysis of mycotoxins and phycotoxins, specific cross-reactivity is calculated after determination of the concentrations of the test substance [TS] and the cross-reacting substance [CS] required for 50% reduction of the absorbance reading compared to that of the zero control standard using the following equation:

$$\text{Relative cross-reactivity, \%} = [\text{TS}] \div [\text{CS}] \times 100$$

However, in the noncompetitive immunoassays, such as the sandwich immunoassay for the analysis of large molecular weight compounds, e.g., allergens, an increase in absorbance reading is used in the calculations. The identification of nonspecific reactivity for some matrixes provide valuable information regarding the extent of applicability of the immunochemical method. Nonspecific cross-reactivity can be determined by a technique described by Morris and Clifford, 1985 (5). This technique quantifies nonspecific cross-reactivity by comparing a standard curve of varying concentration of specific antigen prepared in buffer, with identical curves prepared in varying and increasing amount of sample matrix. If the curves superimpose at all points, then one can assume that the IgG being examined is specific for the antigen in question and is not susceptible to interference from other related compounds (known or unknown), which may be present in the sample matrix.

Recommendation

(a) *Nonspecific cross-reactivity.*—When it has been identified, the occurrence of interferences due to the effect of some matrixes have to be indicated in the scope or applicability of the method. Presence of false positive and/or false negative results for some matrixes have to be clearly mentioned. Consequently, it should be stated under the scope or applicability section in what matrixes (commodities, foods, etc.) the antibody and/or system is intended to be used. Example 1: "This assay or system is intended for use in corn and wheat only. The effect of other sample matrixes on the assay has not been determined. The effect of other sample matrixes can give in-

interferences and, consequently, indicate false positive or negative results." Example 2: "This assay or system is intended for use in corn and wheat only. The effect of other sample matrices such as corn gluten, citrus pulp..., give interferences and, consequently, indicate false positive (or negative) results."

(b) *Specific cross-reactivity*.—All significant specific cross-reactions should be indicated. In case the analyte belongs to a group of compounds, which are structurally closely related, every group members should be tested. For example, for an antibody used in an assay for one or total aflatoxin(s) in cereals, at least AFB1, AFB2, AFG1, and AFG2 have to be tested. However, for some other groups involving many compounds, which can be divided in 2 or several subgroups or families, specificity study should be conducted on the major compound or the family of compounds considered as analyte and its most appropriate closely related congeners. Particular recommendation may be addressed according to both particular group of substances and particular assay design. Tests should be grouped according to their specificity pattern as:

(i) *Cross-reactivity $\leq 10\%$* .—Substance specific; test may be used for quantitative determinations so long as the cross-reacting substance is not present within the sample matrix at levels greatly above those of the test substance (example: even if target compound A and cross-reacting compounds B–D are present in one sample at equal concentrations, the result will be 1.4 times overestimation of A at a maximum, which can be considered to be within the general limits of purpose of enzyme immunoassays). (ii) *Cross-reactivity ranging from >10 to 100%* .—Qualitative test; may be used for quantification only if the identity of the compound is known (example: if compounds A–D are present at equal concentrations, overestimation of A may range from 1.1 to >4 , which cannot be accepted as quantitative). (iii) *Cross-reactivity ranging from ≥ 50 to $\leq 150\%$* .—Semi-quantitative group-specific test; may be used for quantification only if the identity of the compound is known (example: Compounds A–D have cross-reactions of 100, 150, 50, and 100%, respectively. There is a 4-fold overestimation of A at equal concentrations of A–D, but since the detection of the group may be desirable, the quantification is still acceptable). Also, this must accommodate assays for toxin groups or families in which the differing congeners have similar toxicity (example: when a family of target compounds of similar structure and toxicity are bound by the antibodies within the assay, and cross-reaction are within ≥ 50 to $\leq 150\%$ range, the additive or integrative nature of the immunoassay is a major benefit, as the assay can give a measure of "total X toxin family").

One aspect which may have to be considered with natural toxins is the purity of the test standard solutions available.

While some substances, e.g., the major aflatoxins, are available with purities $>99\%$, other toxin standards may contain other with structurally related toxins. In the latter case, the information given by specificity tests has to be taken with care (3).

(c) *Immunoaffinity chromatography columns*.—When antibodies are immobilized in an IAC column, 2 special criteria have to be mentioned: (i) *Selectivity*.—All compounds which are specifically bound by the antibodies used in IAC under the test protocol should be listed. (ii) *Capacity*.—The maximum amount of specific antigen–analyte per column bound under working conditions should be determined and indicated. In the case of multi-analyte column, indicate whether or not and to what extent the presence and binding of analyte A may interfere with binding of analyte B.

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References

- (1) Fremy, J.M., & Chu, F.S. (1989) *Mycotoxins in Dairy Products*, H. van Egmond (Ed.), Elsevier Applied Science Publishers, Barking, UK pp 97–125
- (2) Fremy, J.M., Puech, L., Dragacci, S., & Krys, S. (1999) *Immunological Methods for the Detection of Diarrhetic Shellfish Poisoning Toxins*, Report for the Symposium on Analytical Chemistry of Phycotoxins in Seafood and Drinking Water, AOAC Annual Meeting, Houston, TX, September 26–30
- (3) Märtlbauer, E. (1993) *Basic Principles which Determine the Quality of Immunoassays*, International Dairy Federation, Brussels, Belgium, Special issue No. 9302, p. 137
- (4) Usleber, E., Dietrich, R., Bürk, C., Schneider, E., & Märtlbauer, E. (1999) *Immunoassay Methods for Paralytic Shellfish Poisoning Toxins*, Report for the Symposium on Analytical Chemistry of Phycotoxins in Seafood and Drinking Water, AOAC Annual Meeting, Houston, TX, September 26–30
- (5) Morris, B.A., & Clifford, M.N. (Eds) (1985) *Immunoassays in Food Analysis*, Elsevier Applied Science Publishers, New York, NY, p. 37