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Foodstuffs — Molecular biomarker analysis — Immunochemical methods for the detection and quantification of proteins

Produits alimentaire — Analyse des biomarqueurs moléculaires — Méthodes immunochimiques pour la détection et la quantification des protéines

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 16, *Horizontal methods for molecular biomarker analysis*.

This third edition cancels and replaces the second edition (ISO 21572:2013), which has been technically revised. The main changes compared with the previous edition are as follows:

— the title has been changed to specify that the document is focused on immunochemical protein detection methods;

— an introduction has been added;

— terms, definitions and references have been updated;

— the text has been modified to improve the document's applicability to general protein analysis applications.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Analytical techniques based on highly specific immunochemical-binding interactions have become key tools for analysing many different chemical and macromolecular analytes, including proteins. Methods utilizing these techniques are widely accepted in the scientific and regulatory communities. Immunochemical assay methods are most commonly used to detect (presence or absence) and/or quantify specific protein analytes such as allergenic proteins, disease marker proteins or newly expressed proteins in biotech crops.

Prior to analysis, samples generally need to be ground or processed in a manner that facilitates extraction of the analyte from the sample matrix. An important step in analytical method development is therefore the selection of a suitable extraction buffer that does not interfere with the analytical method performance and that ensures an appropriate level of analyte stability during the analytical process.

The immunochemical assay process generally incorporates at least two steps:

— binding or capturing the analyte of interest present in samples with an antibody targeted specifically to the analyte;

— detection of the antibody-analyte complex using a technique that signals the specific interaction.

Once an analytical method has been developed and optimized, it should be validated to demonstrate that its performance is reliable and suitable for the intended use and to characterize the method limitations. This involves performing several experiments with real samples to evaluate parameters such as accuracy, precision, sensitivity, selectivity and the detection or quantification limits. Validation also allows for the establishment of method performance criteria, against which routine analytical performance can be compared to ensure that acceptable analytical results are consistently reported.

This document provides a set of general procedures and analytical considerations for using immunochemical techniques to analyse target proteins. It discusses aspects of sample processing, extraction, assay set-up, interpretation and reporting of results, and relevant assay performance parameters. Two annexes are included containing example procedures that can be followed when analysing a protein of interest (POI) in a variety of background matrices using methods based on enzyme-linked immunosorbent assays (ELISAs) and lateral flow devices (LFDs).

Foodstuffs — Molecular biomarker analysis — Immunochemical methods for the detection and quantification of proteins

1 Scope

This document specifies performance criteria for immunochemical methods for the detection and/or quantification of a specific protein or protein(s) of interest [POI(s)] in a specified matrix.

The methods discussed are applicable to the analysis of proteins from a variety of sample types. Some uses for these methods include, but are not limited to, analysing proteins involved in crop and food production, food processing, food marketing, food safety, biotechnology or disease indexing.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 16577, *Molecular biomarker analysis — Terms and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 16577 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
conjugate
material produced by attaching two or more substances together by covalent bond via chemical groups

Note 1 to entry: Conjugates of antibodies with fluorochromes (e.g. chemical entity, such as a molecule or group, that emits light in response to excitation by absorbed incident light), radiolabelled substances, gold or enzymes are often used in immunoassays.

4 Principle

The target protein is extracted according to the procedure described for that specific matrix, and a specific antibody is used to detect or measure the concentration of the POI in the sample. For the detection of specific proteins in ingredients, the basic principle of a protein-based method is to:

— take a representative sample of the matrix;

— extract the proteins;

— detect and/or quantify the specific protein derived from the matrix under study.

5 Reagents

During the analysis, use only reagents of recognized analytical grade and only de-ionized or distilled water or water that has been purified, or equivalent unless indicated otherwise by the manufacturer of the reagents or the kit.

Other reagents, such as antibodies, conjugates, substrates, stop solutions and buffer components are method specific. Refer to the method for specifics regarding reagents such as protein standards or reference materials, antibodies or pre-coated solid surfaces, controls, and samples.

Reagents are specified in A.4.2, A.4.3, B.4.2 and B.4.3.

6 Laboratory equipment

Laboratory equipment is specified in A.5 and B.5.

7 Sampling

Sampling is not part of the method specified in this document, though Annex A and Annex B do provide sampling instructions as per the relevant methods. It is recommended that the parties concerned come to an agreement on this subject.

8 Procedure

8.1 General

Storage conditions and the shelf-life of LFDs, antibodies, conjugate, substrate, etc. shall be clearly specified by the provider.

Use appropriate laboratory equipment with low protein binding capacity (e.g. polypropylene tubes) to prevent protein adsorption during the whole procedure.

For the use of this document, general requirements of quality assurance for laboratories shall be observed (e.g. concerning calibration of apparatus, double determination, blanks, use of reference materials, preparation of calibration curves) Carefully clean all equipment coming into direct contact with the sample to prevent contamination. See ISO/IEC 17025 for more information.

8.2 Preparation of sample solution

Once a representative sample is obtained, prepare the sample solution. Sample preparation procedures are described in Annexes A and B.

Grind samples as specified in the method before test portions are taken, if necessary. Powders/flour can have swelling properties and could require more extraction solution if a manufacturer's method does not specify this information. If the sample is not immediately used, follow the laboratory's procedure for storage (e.g. –20 °C or below).

Weigh an appropriate amount of a representative test sample (as specified in A.6.6.1 and B.6.2.1) for analysis to create a test portion for extraction. Add extraction solution and homogenize or mix.

Laboratory samples containing high amounts of fat can be non-homogeneous and a larger test portion should be extracted to ensure that it is representative. If applicable, instructions can be found in the sample preparation sections of Annexes A and B.

8.3 Extraction

Use an extraction procedure suitable for the matrix. Details of appropriate conditions for the extraction/dilution of the test portions, controls and reference materials are provided in Annex A for ELISA and Annex B for LFDs. Care should be taken to use extraction procedures validated for the matrix. Extracted samples should be immediately used or treated as specified in the procedure for storage.

8.4 Preparation of calibration curves, positive controls, and reference materials

For the preparation of calibration curves, positive controls and reference materials for Annex A, it is recommended to use matrix-matched reference materials or reference materials that have been validated for the matrix. Calibration curves are not required for qualitative application such as LFDs. However, positive and negative controls can be prepared at the discretion of the analyst.

8.5 Assay procedure

For a quantitative test, select the required number of wells, (e.g. in ELISA) for the test portion(s) to be analysed, including blanks, positive controls and negative controls, and add each of them, at minimum in duplicate and properly diluted so as to be within the range of the assay.

For a qualitative test or semi-quantitative test, select the required number of tests (e.g. ELISA or LFDs) needed for the test portions to be analysed, including blanks, positive controls and negative controls. The stability of the final signal can vary. Read the results in a timely manner as specified in Annexes A and B.

According to the method chosen, follow the instructions of each method for sample analyses, including blanks, reference materials and/or measurement standards (if necessary). Allow the reaction to occur at a specified temperature range and time. If necessary, terminate the reaction according to the method described in A.6.6.2.7 and B.6.4.2. For example, if the ELISA method requires acquiring data on a spectrophotometer, perform this step. In the case of qualitative tests, follow the kit instructions. Generally, these are interpreted visually.

9 Interpretation and expression of results

9.1 General

The parameters to interpret vary depending on whether the assay is qualitative, semi-quantitative or quantitative.

For quantitative methods, the coefficient of variation (C_V) of optical density values resulting from replicate measurements of a sample test solution, in general, should not exceed 15 %. The coefficient of variation of calculated concentrations resulting from replicate measurements of a sample test solution, in general, should not exceed 20 %.

If the coefficient of variation limit is exceeded, the analyses should be repeated on freshly prepared sample test solution. To establish a coefficient of variation, in this case, at least three determinations shall be carried out (e.g. values from three micro-titre wells).

Negative results shall be reported as “negative at the limit of detection” and the limit of detection (LOD) shall be reported.

Positive results below the limit of quantification shall be reported as “positive above the limit of detection, but below the limit of quantification”. The limits of quantification and detection shall be reported.

9.2 Quantitative and semi-quantitative analysis

For quantitative and semi-quantitative analysis, the following parameters shall be evaluated:

— raw data of the sample test solution;

— blanks;

— reference materials or measurement standards;

— negative controls;

— % C_V between replicates;

— % C_V of standards;

— % C_V of control samples.

In accordance with ISO/IEC 17025, measurement uncertainty should be reported, where applicable.

Quantitative results shall not be reported by extrapolating above the highest or below the lowest calibration point.

9.3 Qualitative analysis

For qualitative tests, including all applications thereof, the corresponding parameters are described in Annexes A and B. The LOD shall always be reported. Negative results shall be reported as "negative at the limit of detection".

Positive results shall also report the LOD.

10 Specific parameters that can influence results

10.1 General

The performance criteria listed in the method of Annex A are a set of performance specifications established for each method during the development, validation and routine use of the method. These parameters shall be estimated and evaluated for each method to ensure they are reliable and of consistently high quality. Each time a method is implemented, the data generated shall be evaluated and compared with the established method performance criteria.

When a value (e.g. coefficient of variation of replicate determinations) does not agree with the assay specifications, it signals that the result is atypical and warrants closer evaluation of the data. The list of specifications shall be taken as whole. In certain instances, individual parameters may not meet the specifications but the data are still perfectly acceptable. If any of the criteria are not met, this should, however, be acknowledged in writing and the data evaluated to determine if the analysis of results should be adjusted, or if a particular sample or a set of samples should be repeated. These decisions should be based on the judgement of the technical expert interpreting the entire set of criteria.

In contrast to the method described in Annex A, the performance criteria of LFD assays as described in Annex B are evaluated during the development of the method by the manufacturer of the kit. The method should be evaluated for repeatability in the laboratory prior to use on test samples. Non-performing kits shall not be used.

10.2 Special considerations

10.2.1 Selectivity

Adequate selectivity of the assay for a particular analyte shall be demonstrated for each POI or analyte (protein) to be measured in each matrix to be tested. Where appropriate, cross-reactivity should be evaluated for analogues (proteins with a similar sequence or structure). To test for the absence of the POI in non-POI sample, assay the non-POI containing sample and POI-containing sample at the appropriate dilutions and compare.

This is generally done during the development and validation of the method and is not necessary during routine analysis of samples for which the method has previously been validated. Selectivity of the test kits, either ELISA or LFD-based methods, should be addressed by the manufacturer of the kit (e.g. listed in the manufacturer's product inserts).

10.2.2 Extraction efficiency

Special care shall be taken to assess the influence of process parameters applied for the production of a given laboratory sample.

In order to provide for the greatest sensitivity of the immunoassay, extraction efficiency should be as high as possible, especially for quantitative methods. The assay performance is matrix dependent. Extraction efficiency should be determined and documented for each matrix.

The extraction procedure shall be demonstrated to be reproducible and the method of calibration (if applicable) should account for incomplete extraction.

10.2.3 Matrix effects

The scope of application clearly and exactly defines the matrices for which the given immunoassay is applicable. The use of matrix matched reference materials allows for direct comparison between reference materials and samples. However, if samples are to be analysed against reference materials that are not the same matrix, then the matrix effects will have to be evaluated.

For example, prepare a negative extract for each sample (matrix) to be analysed by the method and an extract of a positive control of known concentration. Prepare a series of dilutions of the positive control in the negative extract and compare the resulting dose response curve with the calibration curve from the method. If the two curves are different, then there is a matrix effect. Use a matrix that most closely represents the true samples that will be tested. A dilution curve with a positive control of known concentration should also be included as a reference. The shape of the calibration curve should not change due to a matrix effect.

10.2.4 Assay applicability

Food processing will generally lead to degradation or denaturation of the POI, which could result in a substantial change in immunoreactivity. Immunoassays should be evaluated for applicability to the POI in processed products.

10.2.5 Hook effect

In an antibody-based LFD and plate format assay, a hook (saturation) effect could lead to a false-negative result. A thorough demonstration that the working concentration range comfortably covers the practical need of POI test samples is necessary.

10.2.6 Parallelism/linearity

For quantitative analyses, the expected dynamic range of the immunoassay should be explicitly stated in the scope of applications for all matrices covered by it. The relationship of the instrument response to known POI concentration may not be linear and shall be established for each quantitative immunoassay

method by the manufacturer. This relationship is typically hyperbolic if the POI concentration is plotted on a linear scale or sigmoidal with a logarithmic concentration scale. Either a 4-parameter logistic or 5-parameter logistic regression model provides the best fit for an ELISA calibration curve, with the linear portion in the centre of the sigmoidal curve representing the optimal region for quantitative analysis. Other regression models (e.g. linear, quadratic, logit-log, spline-fit, third order polynomial) may also be suitable to fit limited portions of the calibration curve.

A minimum of four calibration points, reflecting the usable portion of the curve, shall be evaluated for quantification purposes, although a 4-parameter logistic fit requires at least five points and a 5-parameter logistic fit requires at least six points.

10.2.7 Limits of detection

Results should not be interpreted below the LOD. In this case, reporting of results shall be stated according to applicable method as described in 9.1 to 9.3.

10.2.8 Limits of quantification

The limits of quantification for each set of calibrants (or dilutions) shall be stated explicitly.

The estimated concentration of unknown sample test solutions shall be interpolated and not extrapolated.

Results shall not be extrapolated below the limit of quantification or above the highest or below the lowest calibration points.

11 Confirming method

To establish the credibility of assays, another method such as western blot, high-performance liquid chromatography (HPLC), mass spectrometry (MS) or a functional assay can be used to measure split analytical samples of known concentration. The results of both methods are then qualitatively/quantitatively compared. This is especially important for immunoassays, since antibodies could cross react with other analytes present in a matrix.

12 Test report

The test report shall contain at least the following information:

a) all information needed to identify the laboratory;

b) all information needed to identify the laboratory sample;

c) reference to this document, i.e. ISO 21572, and to the method used, and an indication of whether it was a qualitative, quantitative or semi-quantitative method;

d) LOD;

e) lower and/or upper limits of quantification;

f) date and type of sampling procedure used (if known);

g) date of sample receipt;

h) analysis start date or other appropriate documentation;

i) amount of the test portion;

j) amount of the test sample;

k) results and the units used to report them;

