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Sharing and promoting Reference Laboratory activities
in Animal and Plant Health, Food and Drinking Water Safety

No.1 - *Tools for reference*

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Editorial

A new life for Euroreference

The first issue of Euroreference (Journal of Reference) was launched in June 2009 as a free online AFSSA [1] publication in both French and English. The original aim was to facilitate the dissemination of information about reference activities among stakeholders both at national and European levels. The topics covered included all scientific and technical aspects of analytical methods and their role in surveillance plans and reference-related regulatory issues. As a common forum for members of reference laboratory networks and all the players concerned, it was geared to National and European Reference Laboratories, animal health and food safety diagnostic laboratories, Ministries and decision-makers, directors of institutions, etc. The AFSSA/ANSES Editorial board was rapidly joined by representatives of CODA-CERVA (Belgium), ISS (Italy) then PIWET (Poland).

In order to consolidate EU efficiency and in the light of likely developments in EU regulations in the area (animal and plant health laws, amendment of regulation (EC) 882/2004, etc.), we consider it essential to broaden the readership and authorship of this communication tool, thus initiating EuroReference's conversion to a truly European journal. This initiative has been enthusiastically received by the sister institutions ANSES managed to visit in 2015 and we are now very pleased to announce the launch of this new formula of EuroReference co-edited by CODA-CERVA (Belgium), ANSES and SCL (France), BfR and FLI (Germany), ISS, IZSAM and IZSLER (Italy), PIWET (Poland), APHA and FERA (UK) in addition to the EPPO. Other EU institutions addressing reference laboratory activities in animal and plant health or food and drinking water safety are of course welcome to take part in this new adventure so as to strengthen the network of EU laboratories working in the sectors of animal and plant health and food and drinking water safety.

This first issue of the new Euroreference formula focuses on "reference tools". We hope you will enjoy it and remain regular readers and authors.

Pascale PARISOT & Bruno GARIN-BASTUJI

1 The French Food Safety Agency (AFSSA) merged with the French Agency for Environmental and Occupational Health Safety (AFSSET) in 2010 to become the current French Agency for Food, Environmental and Occupational Health & Safety (ANSES).

INTER-LABORATORY COMPARISONS

Organisation of proficiency testing and other activities of the European Union Reference Laboratory for chemical elements in food of animal origin (EURL-CEFAO)

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Abstract

The EURL-CEFAO, hosted by the Istituto Superiore di Sanità of Rome (ISS), is one of the European Union Reference Laboratories (EURLs) designated by Council Directive 96/23/EC responsible for chemical elements (group B3c) in food of animal origin.

The tasks of EURLs are listed in Article 32 of Regulation 882/2004. In particular, the EURL-CEFAO focuses its activity on preparation of *ad hoc* reference materials for inter-laboratory comparisons for its network of National Reference Laboratories (NRLs) and validation of easy-to-use methods based on the most widely used techniques in its field of competence. In addition, other activities include organisation of a yearly workshop, assistance to EU-NRLs, and organisation of training courses for NRLs of candidate EU Member States and non-EU countries.

Keywords

- Chemical elements
- European Union
- Food
- Laboratory proficiency testing
- Organisation

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Introduction

In order to ensure the safety of food products and guarantee public health, the European Commission has nominated European Union Reference Laboratories (EURLs) to contribute to the standardisation of analytical methods and the harmonisation of performance among the EU Member State National Reference Laboratories (NRLs). In this way, analytical data should have the same level of quality and reliability in all member states. The tasks of both the EURLs and the NRLs are listed in Regulation (EC) No 882/2004 of the European Parliament and of the Council "on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules". In particular, Article 32 of this Regulation defines the EURLs' general tasks that are mainly focused on technical support to the European Commission in their field of competence and assistance to the NRLs. Therefore, the EURLs act as an interface between the European Commission and the NRLs.

The European Union Reference Laboratory for Chemical Elements in Food of Animal Origin (EURL-CEFAO) is housed in the Department of Veterinary Public Health and Food Safety (ISS) and includes four researchers and three technicians. It is responsible for the substances listed in Annex I, Group B3(c) in Council Directive 96/23/EC of 29 April 1996, *i.e.* Chemical Elements in Food of Animal Origin. The presence of chemical elements in these matrices is not the result of intentional use for animal treatments, but is rather related to environmental contamination as well as contamination of feed and dietary supplements used in animal feeding. The inclusion of these elements in a European Commission Directive regarding residues in live animals and animal products was mainly due to the fact that some legal limits for these substances, considered potentially toxic in humans, had already been established in order to guarantee maximum protection of EU consumers.

The EURL-CEFAO acts to ensure that the National Reference Laboratories belonging to its network uniformly apply Regulation (EC) No 1881/2006 and subsequent amendments, which set maximum levels (MLs) for certain chemical elements in foodstuffs. Close interactions

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between the EURL and the NRLs are a key to improving consistent and homogeneous application, especially since the main task of the NRLs is to increase the quality, accuracy and comparability of the results produced by official control laboratories.

In compliance with the duties listed in Article 32, the EURL-CEFAO carries out a wide range of different activities, but over the years it has focused its work on: method validation based on the most widely used techniques in its field of competence, organisation of proficiency testing (PT) intended for NRLs, implementation of follow-up actions for under-performing laboratories, assistance to the EC and NRLs, and organisation of workshops and training courses. Some aspects of these activities are detailed in this article, pointing out their relevance in food safety policy.

Accreditation Status

The EURL-CEFAO has developed its own quality system on the basis of which it has been accredited as a testing laboratory according to ISO/IEC 17025 since 2005. In order to improve the qualification of its work and promptly face the needs arising from the update of regulations relevant to MLs for food of animal origin, the EURL-CEFAO applied for and obtained the flexible scope of accreditation. Furthermore, the laboratory has been accredited as a proficiency testing provider (PTP) according to international standards since 2010. In particular, the first accreditation as PTP was in accordance with ISO Guide 43-1 and subsequently, on 9 January 2012, the EURL-CEFAO was the second laboratory in Italy to be accredited according to ISO/IEC 17043:2010. This status enables the EURL-CEFAO to provide participants with exercises of recognised high quality.

All the accreditation processes were completed by the Swedish Board of Accreditation (Swedac) but, as a consequence of Regulation (EC) No 765/2008 of the European Parliament (Articles 4 and 39), in July 2012, the EURL-CEFAO successfully transitioned to the national accreditation body ACCREDIA for both ISO 17025 and ISO 17043.

Coordination of the NRL network

The EURL-CEFAO manages a network of 28 NRLs designated by the competent authorities of the relevant EU Member States.

An important tool to coordinate the network and to share information among the laboratories is the EURL website (**http://www.iss.it/lcdr/**). It consists of two sections: an open-access area and a restricted area where it is possible to access confidential information (*e.g.* outcomes and follow-up of PTs, handbooks of NRL analytical methods, etc.). As of 2016, a specific section will be included in the restricted area to better manage proficiency testing. In fact, this new dedicated section will enable the NRLs to confirm their participation in the PT and directly upload their results to the website.

One of an EURL's duties is to organise an annual workshop. The EURL-CEFAO pays special attention to this meeting because it is an important opportunity to share technical experience and to disseminate information regarding analytical methods and PTs. Furthermore, during the workshop a representative of the European Commission provides updates on changes in regulations.

In addition to the NRL network, several laboratories from other countries such as Brazil, Denmark, FYROM, Luxembourg, Norway and Russia participate in the inter-laboratory compari-

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son and some of them attend the annual workshop.

This is evidence of the increasing interest of many countries in the activities of the EURL-CEFAO.

Cooperation with other EURLs and organisations

The EURL-CEFAO has been collaborating with the EURL for Heavy Metals in feed and food (EURL-HM) for many years. The objective of this collaboration is to exchange views and proposals about topics of interest to NRLs operating in the field of chemical elements in food. Furthermore, the EURL-CEFAO is in contact with the EURL for Single Residue Methods (EURL-SRM) located in Stuttgart, Germany operating in the field of pesticide residues. In particular, cooperation is focused on organising proficiency tests on the determination of mercury and copper in meat.

The EURL-CEFAO's director is also a member of the EURACHEM PT working group. Moreover, the EURL's experts provide their technical opinion to the Italian Ministry of Health so as to support the Italian position when the revision of maximum levels for some matrix/element combinations is discussed at the European Commission level.

Activity as a proficiency testing provider

The EURLs play a key role in guaranteeing that analytical results produced by the NRLs are as reliable and uniform as possible. The EURL-CEFAO is aware of how valuable PTs are in checking and harmonising laboratory performance. To this end, the laboratory has invested significant energy and resources in the organisation of inter-laboratory comparisons in order to reach the required quality and uniformity of analytical results produced within the European Union.

In particular, to better qualify this activity, the laboratory has been accredited as a PTP since 2010 and over the years it has developed and optimised many procedures to produce materials suitable for its tests. Except for a few specific processes (*i.e.* sterilisation and lyophilisation) performed by qualified suppliers, all steps of PT sample preparation are carried out by the EURL staff in the laboratory's facilities. Furthermore, EURL staff performs the statistical evaluation of the participants' results under a scheme based on the consensus approach. Therefore, thorough knowledge of every aspect of PT organisation allows the EURL-CEFAO to know the drawbacks of each phase and the best ways to overcome them.

As far as the programme is concerned, the number of PTs to be conducted each year as well as the matrix/analyte combinations on which they are based are to be set in the annual work-programme submitted by the EURL to the European Commission. In this schedule, both the outcome of the previous rounds and the specific needs of NRLs are carefully evaluated.

The PT material is usually prepared starting from commonly consumed food of animal origin purchased at retail stores. The concentration levels of analytes of interest (*e.g.* As, Cd, Pb, Hg, Mo, Cu) are often adjusted so as to be around the MLs set in the relevant regulations. As for the physical state of the matrix, considerable efforts have been made over the years to distribute fresh test items (*e.g.* frozen meat, frozen fish, frozen offal) or liquid test items (*e.g.* milk) [Ciprotti *et al.*, 2013] instead of freeze-dried samples [Colabucci *et al.*, 2015]. In fact, although lyophilised matrices are easier to handle and store, the fresh or liquid items are more representative of the real samples that laboratories are called on to analyse during their routine work. Moreover, sample compliance has to be stated on fresh foodstuffs for which MLs

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are set in the pertinent legislation. Therefore, the inclusion in the EURL-CEFAO scheme of matrices in this physical state is particularly useful to the NRLs.

Starting from 2006, the EURL-CEFAO focused its activity as a PTP on improving and harmonising the performance of its network on the analysis of chemical elements in matrices for which MLs are established in Commission Regulation (EC) No 1881/2006 and subsequent amendments.

After this initial period, as the network reached an extremely satisfactory and steady level of performance, the EURL-CEFAO focussed on other matrix/element combinations, in particular those included in the National Residues Control Plan that each Member State submits annually for approval to the EC. This plan contains information such as the species to be controlled, the analytes to be determined, the number of samples, the sampling procedures, the requirements for laboratories performing the analyses, and the actions to be carried out if samples are not compliant.

TABLE 1 / Proficiency tests organised by the EURL-CEFAO over the period 2006-2015. Analyte/matrix combination and physical state of the samples.

Year	EURL-CEFAO's PTs		
2006	Meat Cd, Pb Freeze-dried	Milk Cd, Pb Freeze-dried	
2007	Meat Cd, Pb Freeze-dried	Milk Cd, Pb Freeze-dried	Fish Cd, Pb, Total Hg Freeze-dried
2008	Milk Cd, Pb Liquid	Liver Cd, Pb Freeze-dried	Meat Cd, Pb Freeze-dried
2009	Fish Cd, Pb, Total Hg Freeze-dried	Milk Cd, Pb Liquid	Fish Cd, Pb, Total Hg Frozen
2010	Meat Cd, Pb Frozen	Milk Cd, Pb Liquid	
2011	Liver Cd, Pb Frozen	Meat Cd, Pb Freeze-dried	
2012	Milk Cd, Pb Liquid	Infant formula Cd, Pb Powdered	
2013	Meat Cd, Cu, Pb, Total Hg Frozen	Honey Cd, Pb	
2014	Kidney Cd, Cu, Pb, Total Hg Frozen	Fish Cd, Pb, Total Hg Freeze-dried	
2015	Infant formula Cd, Pb, Mo Powdered	Mussels Total As, Cd, Pb, Total Hg Freeze-dried	

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This strategy has enabled the EURL-CEFAO to gain a clear picture of the matrices of increasing interest, such as honey [Ciaralli *et al.*, 2015] and infant formula [Sorbo *et al.*, 2015], so as to organise exercises before the relevant MLs become applicable. This gives the participants the chance to address analytical issues related to these matrices in advance. The PTs organised in the ten-year period 2006-2015 are summarised in table 1.

In addition, the inter-laboratory comparisons are designed as part of a long-term programme pursuing the objective of providing the participants with a scheme useful for checking and improving the performance of their analytical methods as well as verifying the effectiveness of any corrective actions in case of under-performance. This objective is met by repeating PTs on the same or similar matrices and/or by producing additional samples to be distributed to the network.

According to international standards, the performance of participants is assessed in terms of z-scores expressed as $z = \frac{(x_i - x_{pt})}{\sigma_{pt}}$ where x_i is the laboratory result, x_{pt} is the assigned value and σ_{pt} is the standard deviation for proficiency assessment. In the food sector the σ_{pt} commonly used is based on the Horwitz/Thompson equation, but, considering that the performance expected from the NRLs should be better than that of routine control laboratories, adequate lower values of standard deviation were set for the proficiency assessment (σ_{pt}). These values are calculated according to some specific equations developed by the EURL-CEFAO.

Over the period from 2005 until now, the general performance of the network has been satisfactory on the whole and in some cases it has improved over the years, even when new Member States entered the network.

The number of exercises carried out in the period 2005-2015 (*i.e.* 24 PTs), as well as the huge amount of samples produced (*i.e.* about 3200 samples) make the organisation of PTs a core activity of the EURL-CEFAO. Furthermore, the high value of the exercises proposed is confirmed by the fact that requests from NRLs not belonging to the EURL-CEFAO network to participate in the PTs have increased over the years (Figure 1).

FIGURE 1 / Increase in the number of participants in EURL-CEFAO proficiency tests organised from 2005 to 2015.

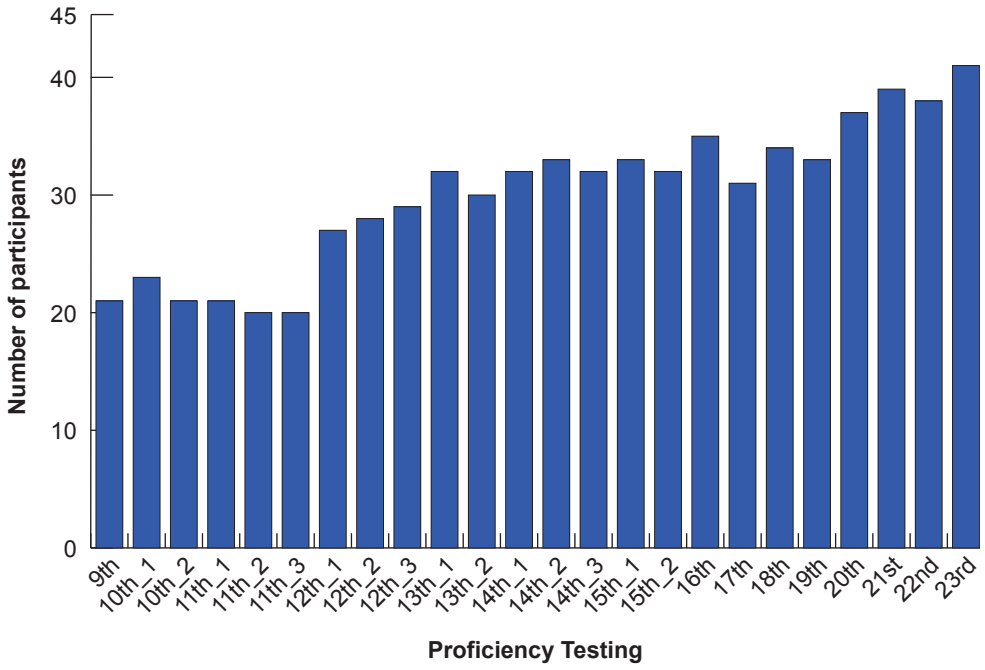

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FIGURE 2a / Percentage of incorrect, not stated and correct assessments of sample compliance with respect to each proficiency test.
Sample compliance for Cd

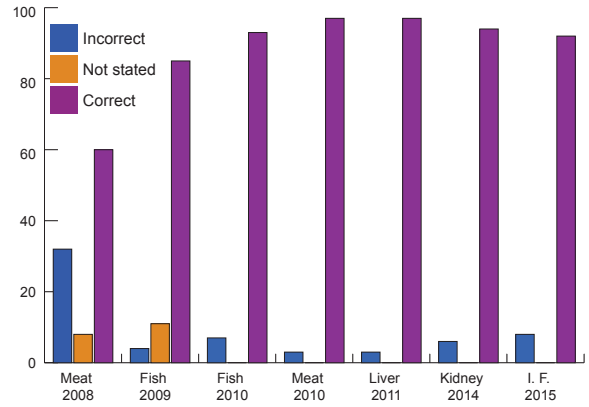
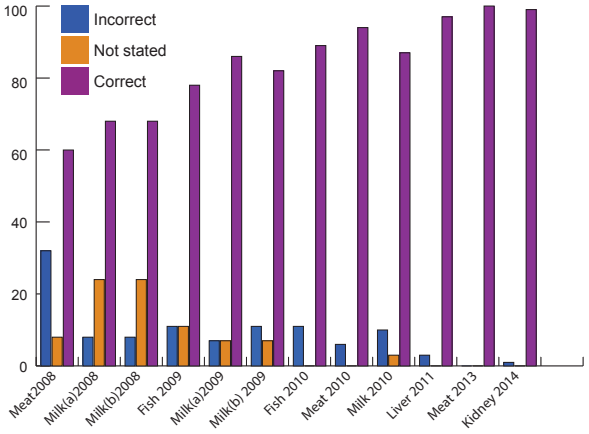


FIGURE 2b/ Percentage of incorrect, not stated and correct assessments of sample compliance with respect to each proficiency test.
Sample compliance for Pb



Considering that NRLs are often appointed as third parties in legal disputes, the exercises proposed by the EURL-CEFAO can be regarded as one of the most useful and proficient schemes available. In fact, other schemes on the market often do not cover matrices of interest, do not propose samples in a physical state similar to routine test items, and do not provide concentration levels suitable for testing methods around MLs. As an example of this deficit, the EURL-CEFAO recently organised a PT (22nd PT) on the determination of cadmium and lead in powdered infant formula and, since a similar exercise was not commercially available, the German NRL asked the EURL-CEFAO to provide them with some samples to be used for a PT involving national control laboratories. On this occasion, the performance of these official laboratories was evaluated on the basis of the assigned values set by the EURL-CEFAO, according to an internal procedure of statistical evaluation and derived from the results submitted by the 28 expert laboratories belonging to the EURL network. This kind of assistance, organised so as not to use EC funds, was also provided to the Italian and French NRLs and can be considered an important tool for harmonising the performance of EU laboratories, even at the local level.

Concerning the sample compliance statement, uniformity of assessment in different EU Member States is an important point to support the EU market. In fact, it is preferable that the same sample be considered in the same way (compliant or not compliant) in different EU Countries. Therefore, in order to ensure uniform assessment, starting from 2008, the EURL-CEFAO requested that participants state sample acceptance according to Commission Regulation (EC) No333/2007, considering this statement as an integral part of the exercise. Sample compliance is requested only for matrix/analyte combinations for which MLs have been established.

The general outcome showed that this issue needed to be addressed especially taking into account the key role played by NRLs. In fact, when this kind of exercise was proposed for the first time, only a few NRLs included the compliance statement for the sample in their results and incorrect assessments were common. The high number of PTs carried out over the last seven years and the fact that discussions on this issue have taken place several times during the workshops, has helped to overcome this problem although some errors still occur (Figure 2).

Evaluation of the long-term performance of NRLs and follow-up and training activities

The EURL-CEFAO monitors the performance of NRLs through a Shewart Control Chart of z-scores [Sorbo *et al.*, 2013], which are prepared for every laboratory, updated after each exercise, and published in the restricted area of the EURL-CEFAO website. Every graph is thoroughly evaluated in order to detect not only unsatisfactory results (|z|≥3) but also trends

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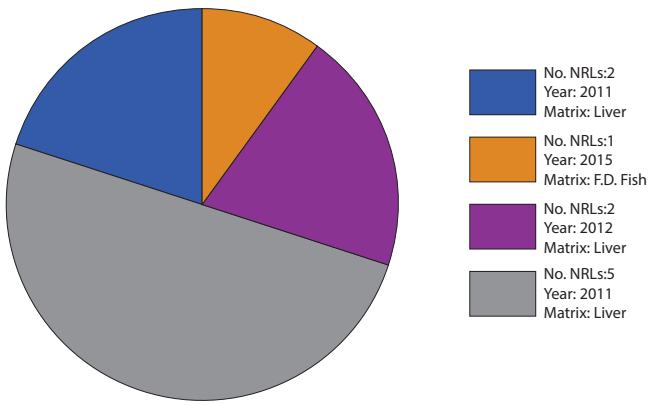
evident in the charts. The outcome of this investigation can prompt specific follow-up actions, which are performed by giving the laboratories suggestions or advice based on the EURL analytical background as well as on analytical information supplied by the participants (*e.g.* sample treatment, dilution factor, calibration points, LoD, LoQ, etc.). Furthermore, laboratories are contacted and requested to inform the EURL about the cause of their under-performance and/or if corrective actions have been undertaken. Should the participants require special analytical support, the EURL staff organises training courses at its facilities or includes this activity in the visits to NRL laboratories. In particular, the laboratories to be visited are selected on the basis of the following criteria: under-performance, new laboratories that entered the network or under-performing laboratories that have changed their analytical technique and laboratories already visited that still demonstrate the need for additional targeted training. In ten years of activity, EURL-CEFAO representatives have carried out 22 training sessions and visits to EU NRLs in order to provide the required support. Even though this specific activity is carried out to accomplish one of the EURL's tasks, the EURL-CEFAO pays a special attention to it, taking advantage of the strong analytical background of its staff. In fact, the possibility of making practical suggestions to NRLs on the way to overcome their analytical obstacles has been found to be the best approach to make the visit beneficial.

Similarly, the EURL organises training courses for official laboratories of candidate Member States and non-EU countries, upon request. Training courses for laboratories in the Philippines, Jordan, Brazil, and Macedonia have been carried out hosting about 15 scientists.

As a further way to address under-performance, the EURL-CEFAO is able to provide NRLs with *ad hoc* samples that laboratories are requested to analyse as if they were PT test items. The submitted results are evaluated by the EURL expert and an individual report containing z-scores and technical comments is sent to the participants. This activity is particularly useful to check the effectiveness of the corrective actions that under-performing laboratories have implemented. Until now, the EURL-CEFAO has organised 10 additional exercises to assist its network as shown in figure 3.

FIGURE 3 / Additional exercises organised by the EURL-CEFAO



Development of analytical methods

The development of new analytical methods is an important part of the EURL's activity [Sorbo *et al.*, 2014]. In particular, two objectives are pursued when the development of a new method is planned: provide the NRLs with analytical procedures suitable for the new matrix/analyte combinations on which EURL-CEFAO proficiency tests will be based and to develop new

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easy-to-use methods that can be required to address emerging topics. In both cases, the results of this activity are shared with the NRLs as these procedures are usually distributed to them or made available upon request. The possibility of developing methods using all the techniques available to the NRLs is one of the strong points of EURL-CEFAO analytical activity. In fact, two different methods are usually developed for the same analyte/matrix combination: one based on inductively coupled plasma-mass spectrometry (ICP-MS) and one based on graphite furnace atomic absorption spectrometry (GFAAS). Furthermore, EURL-CEFAO experts have validated methods for mercury determination based on both cold vapour atomic absorption spectrometry (CVAAS) and direct mercury analysis (DMA).

The methods always comply with the performance criteria required in Commission Regulation (EC) No 333/2007 and subsequent amendments. Over the years, 12 methods have been distributed to the NRLs, including a new method for inorganic arsenic determination in fresh mussels using water bath extraction and anion exchange chromatography-ICP-MS. This procedure is of particular interest to the laboratories as the concentration of inorganic arsenic in foodstuffs is a major concern for public health.

Conclusions

The strong connection between the EURL and NRLs enables easy sharing of analytical experience and development of expertise in the analytical sector within the European Union. The EURL-CEFAO carries out its activity with the aim of not only fulfilling all its duties but also being as useful as possible in the implementation and maintenance of high quality standards required by laboratories that have been appointed as NRLs.

Regulations on food safety control as well as analytical techniques are constantly evolving. As a result, the EURL-CEFAO needs to continuously update its technical knowledge. Over the years, this picture has led the laboratory to reach an ever-increasing level of specialisation in the activities carried out in order to be able to disseminate its experience among the NRLs. The activity performed by the EURL-CEFAO has received positive feedback from the EC and the NRL networks, confirming the suitability and effectiveness of the strategy that the laboratory follows in planning its work.

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Advantages of proficiency test items utilising real food matrices

Mark Sykes*, Ken Mathieson, Dominic Anderson, Emma Hutton, Michael Knaggs**.

Abstract

Proficiency testing by interlaboratory comparison requires standardised test items to be distributed to all the participants. Since the participant laboratories are routinely receiving a variety of food types for analysis, a simple matrix test item would be more straightforward to produce.

However, this does not then reflect the types of samples received by the laboratory, which is a highly desirable element of the test. Real food matrix test items reflect not only the types of samples being routinely received and analysed by the laboratory but also the effect of critical method or preparation parameters. Several examples from a food analysis proficiency testing provider detail the advantages of issuing real food matrix test items. The effect of instructions to participants, their applied methods and critical parameters, and the additional benefits of incurred or contaminated materials are demonstrated.

Keywords

- Chemicals
- Food
- Laboratory proficiency testing
- Matrix
- Microbiology

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Introduction

Proficiency testing (PT) aims to objectively assess a laboratory's performance against a standard test item. In its simplest form, a standard solution (for analytical chemistry measurements) would serve this purpose. However, laboratories in the real world are not actually measuring standard solutions; they are measuring real samples made of complex matrices. A good proficiency test sample has to replicate, as far as possible, these real world samples. At the same time, the test sample has to be the same for each participating laboratory (which may be hundreds of laboratories).

A good complex matrix for a proficiency test item is one which inherently incorporates the analyte within its chemical structure, for example fat in processed food or veterinary drug residues in tissues. An alternative type of test item is one that contains the food matrix plus a separate standard of the analyte, which then has to be mixed by the participant prior to analysis. This is less desirable (since it does not replicate a real world sample) but has its advantages for the PT provider in terms of preparation and, possibly, performance assessment.

When considering a proficiency test item, the PT provider needs to know something about the methods that will be applied in its analysis. This is essential information because the results might be method-dependent and most food PT schemes set the assigned values on the basis of the participants' results. Fortunately, many methods can be applied to give the same answer (within defined acceptance criteria) but, even so, there might be a common parameter which is essential to be followed. A hydrolysis step for total fat determination is one example (discussed later).

A desirable feature of a PT scheme [ISO/IEC 17043:2010] is to capture information on participants' methods. This serves three purposes. First, it enables the PT provider to accept results from any method (so not to dictate to the participant which method to use). Secondly, it permits the PT provider to assess the results against specific method parameters, if there is evidence in the distribution of method-dependency. Thirdly, it allows participants to compare their method with those of the other participants to verify that a particular method works (or does not work).

Method dependency has a number of sources but they can broadly be categorised into:

- initial sample preparation,
- sample extraction,
- sample clean-up,
- and determination.

The first of these is often neglected in a PT because the samples are all homogenised (standardised) before dispatch to participants. Nevertheless, instructions to participants should still define how the sample is to be prepared initially, which often includes appropriate storage conditions and to mix the sample thoroughly before taking the analytical test portion. The determination step is less likely to be a major source of method dependency, since by this time the matrix effects will have been mitigated by appropriate sample extraction and clean-up procedures. In fact, it is the proper application of extraction and clean-up that is often critical to the success of a PT using complex matrix test items.

There are few exceptions and, for the sake of completeness, it is worth mentioning one. A PT for food contact materials necessarily standardises across a defined method [EN 1186-1:2002] and food simulant. The reason for this is that there is no extraction or subsampling, so the primary source of variation is the conditions under which the test is undertaken (time and temperature). This needs to be standardised for all participants for the test to be comparable.

This paper relates the range of method responses to results recorded in some FAPAS PTs

and discusses the advantages of utilising real food matrices from the perspective of the participants and the provider.

Previous examples of matrix or method dependency

It is not the purpose of this paper to provide a wide-scoping review of PT schemes that have found matrix or method dependency. However, it is worth highlighting two dependencies previously published by FAPAS.

■ Pesticides spiking

Many laboratories rely on formulation to provide an estimate of extraction efficiency or recovery. Typically, this involves spiking a blank matrix subsample with a known quantity of analyte immediately prior to analysis. While this approach is acceptable for immediate quality control purposes, it fails for pesticide residues PT materials. A number of weeks or months may elapse between the preparation of the test material and the actual running of the PT. During this time, pesticides may bind to the matrix such that there is a significant difference between theoretical spike values and actual consensus assigned values (and homogeneity mean values). This difference was determined [Sykes *et al.*, 2013] to be systematically a factor of 1.22 of spike value to assigned value.

■ Vitamins

Vitamin analysis has notable method dependencies that might not be apparent from an internal validation exercise. It is only when an interlaboratory comparison is applied that such method dependencies become visible across a large population of laboratories. One such example is that of vitamin B2 specifically in liquid supplement [Sykes *et al.*, 2013]. Due to the differing solubility, in a liquid supplement vitamin B2 is mostly in the form of riboflavin 5'-phosphate, not riboflavin. This requires a two-stage extraction process, firstly applying the common acid hydrolysis and, secondly, an enzymatic hydrolysis to release the phosphate. There is a clear and quantifiable distinction between those laboratories applying only the acid hydrolysis step and those applying both acid and enzymatic hydrolysis steps.

Instructions to participants

■ Total fat hydrolysis, example

One advantage of a PT is that participants generally are free to use their own routine method and this is not specified by the PT provider. However, as noted above, there remain some specific method parameters that might influence the results and the outcome of the PT. If they are critical, these need to be taken into account at the outset of the PT. In nutritional components analysis, the total fat determination is dependent on the use of a hydrolysis step. Although participants are still free to use their own method, an advisory instruction might be included relating to the use of valid data in the calculation of the consensus assigned value. One such example comes from FAPAS PT 25131 [FAPAS Reports], total fat in fish paste. The instruction to participants states, 'Please state if you have used acid hydrolysis or not. Results for total fat will only be included in the assigned value calculation if the use of acid hydrolysis is reported.' Out of 55 results reported for total fat, 32 (58%) claimed that acid hydrolysis was used in the extraction procedure and the assigned value (robust mean, 16.89 g/100 g) was calculated using only those data. If we take the data which haven't applied acid hydrolysis,

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the median value is 16.12 g/100 g. The median value of the acid hydrolysis data is also 16.89 g/100 g, and this is significantly different (P-value 0.0021 at 95% confidence). The observed distributions for the two separate sets of data are both symmetrical but when combined show a distinct skew (Figures 1a and 1b).

FIGURE 1a / Histogram of PT z-scores for total fat in FAPAS PT 25131, assigned value set using only data reporting the use of acid hydrolysis.

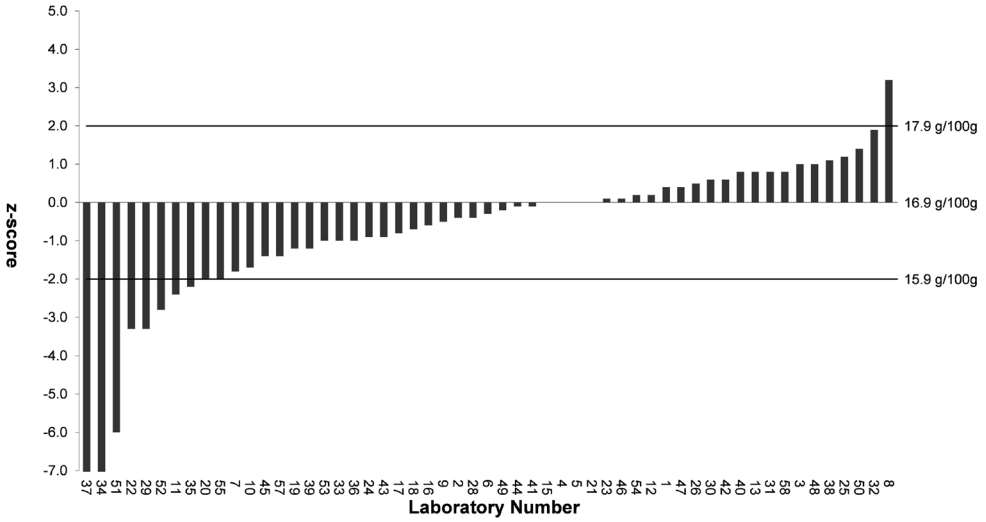
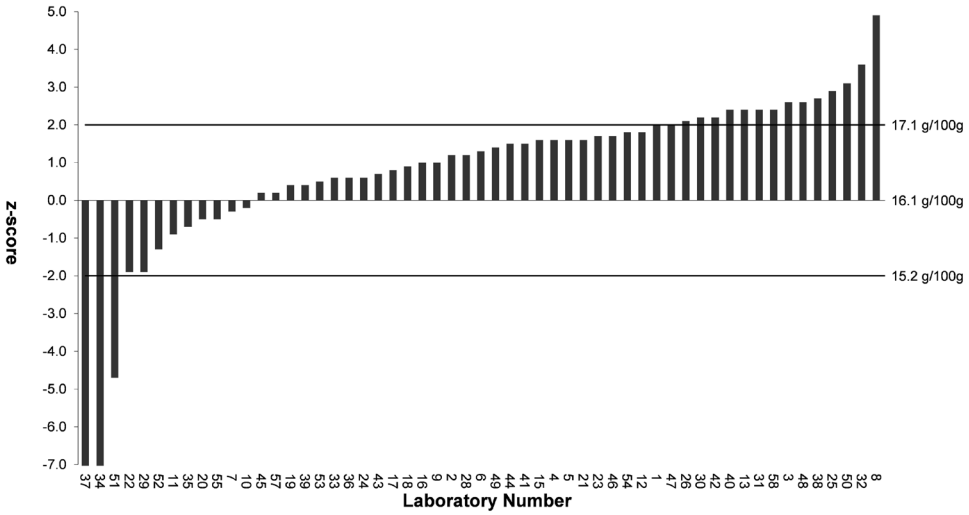


FIGURE 1b. Histogram of PT z-scores for total fat in FAPAS PT 25131, assigned value set using data not reporting the use of acid hydrolysis.



■ Food Microbiology, example

The FAPAS food microbiology PT samples are real food matrices incorporating the organisms and freeze-dried. Thus, the organisms are inherently part of the matrix which requires reconstituting before analysis. Instructions [FAPAS Reports] are provided to participants with regard to sample reconstitution and will typically include details as follows:

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Meat, egg, rice and green vegetable samples require rehydration in buffered peptone water (10 ml or 25 ml, depending on the test). The whole sample requires rehydration in the container provided and not be further subsampled. After mixing the buffered peptone water, the sample is left to stand for 30 min and is then ready for analysis, as a 10 or 25 g equivalent real sample. The use of buffered peptone water matches the initial diluent already used by the majority of laboratories.

Milk powder and animal feed samples require a larger volume of the laboratory's own diluent or pre-enrichment broth to give a reconstituted sample equivalent to 1/10 dilution of a real sample. The whole sample must be reconstituted in an appropriately large container or homogeniser bag, ensuring that the entire sample supplied has been transferred. A 30 min equilibration period is required.

Soft cheese and dry powder samples (black pepper, cocoa powder, flour, infant formula) are analysed without reconstitution. However, the whole sample must again be taken, not a subsample.

TABLE 1 / Target and background flora from PT FEPAS 202.

Proficiency test	Matrix	Target organism	Background flora
Enumeration of coliforms	Beef	*Escherichia coli*	*Micrococcus luteus* *Staphylococcus aureus* *Citrobacter freundii*
Enumeration of *Escherichia coli*	Beef	*Escherichia coli*	*Proteus vulgaris* *Micrococcus luteus* *Enterobacter aerogenes*
Enumeration of *Clostridium perfringens*	Milk powder	*clostridium perfringens*	*Bacillus coagulans* *Staphylococcus aureus* *Bacillus cereus*
Aerobic plate count and Enumeration of *Bacillus cereus*	Milk powder	*Bacillus cereus* *Lactobacillus plantarum* *Pseudomonas aeruginosa*	not applicable
Detection of *Salmonella* spp. test material A	Chicken	not applicable	*Citrobacter freundii* *Staphylococcus aureus* *Proteus vulgaris*
Detection of *Salmonella* spp. test material B	Chicken	*Salmonella Cerro*	*Citrobacter freundii* *Staphylococcus aureus* *Bacillus cereus*
Detection of *Campylobacter* spp. test material A	Chicken	*Campylobacter jejuni*	*Pseudomonas aeruginosa* *Bacillus cereus* *Proteus mirabilis*
Detection of *Campylobacter* spp. test material B	Chicken	not applicable	*Pseudomonas aeruginosa* *Bacillus cereus* *Proteus mirabilis*

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Real world samples arriving at the laboratory will not, generally, require this additional preparation procedure; they would be analysed as received. However, for the purposes of providing a stable and homogeneous test material, this slight difference to a real world sample is necessary. The matrix is real food, not a simulant, and when reconstituted is effectively indistinguishable from a homogenised wet food. Dry powder samples are, of course, identical to the matrix of a real world sample.

A further complication in the test material might be to deliberately introduce background organisms. A real world sample will inevitably contain other organisms in addition to the ones being targeted by the analysis, so why not replicate this in a PT sample? This is highly desirable because the correct target organisms need to be identified before enumeration. Even in a simpler detection test, a decision needs to be made by the participant on which of two test items is positive with regard to the organism being detected. A simple PT which is only positive with regard to the target organism and includes one completely blank sample (no organism at all) would not make for a very effective PT. An example of target and background organisms from a typical FAPAS food microbiology PT is provided in Table 1

Participants' reported methods

Proficiency tests differ from collaborative trials in that PT participants generally are free to use their own method of choice. This will be the method in routine use in their laboratory and, usually, the one that they hold accreditation for. Collaborative trials, by their very nature, are method-specific with carefully controlled and defined parameters. This might even dictate the exact chromatographic column to be used. The PT method of choice often will have been based on a previously published method, perhaps even an international standard. However, these are often modified by the laboratory to suit their particular circumstances, so rarely is a well-defined method followed to the letter. This in turn means that simply capturing a method reference is inadequate and that capturing specific method parameters is of more use to anyone with an interest in the PT report.

How much detail is required or useful to the reader is open to debate. Taking a simple moisture determination as an example reveals the potential complexity. The FAPAS PT 25131 (nutritional components in a fish paste test material) [FAPAS Reports] for moisture revealed that some 32 participants returned method details, out of 57 results. The method details are summarised in Table 2 (at end of article). (NB. returning method details is not a mandatory part of a PT, so will inevitably be incomplete.) The sample weight data demonstrates the scope for variation, with two laboratories taking between 1 and 2 g subsamples, 13 laboratories 2-5 g, 15 laboratories 5-10 g, and 2 laboratories 10-25 g. Of the 8 laboratories reporting that they follow an international standard method, 5 are in the 5-10 g category, 2 laboratories are in the 2-5 g category and one laboratory in the 10-25 g category. An order of magnitude range in subsample size for a PT sample is less likely to have an impact than a real world sample which is not proven to be homogenous. Nevertheless, if a PT sample is to replicate real world samples, methods applied in a PT ought to consider real world variables.

A further example highlights a difference within pesticide residues testing, which has seen a lot of development in recent years towards generic extraction methods. Methods of analysis for pesticide residues can broadly be categorised into those applying gas chromatographic separation (GC) or liquid chromatographic separation (LC). The extraction solvent of choice tends to match the chromatographic method, with acetonitrile being the common choice for LC and acetonitrile, acetone or ethyl acetate the solvent for GC (as a generalisation). This generalisation tends to apply regardless of the matrix and reflects the affinity of the pesticides being sought for GC or LC approaches.

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However, where cereals or their products are being analysed, either method benefits from pre-treatment of the sample with water. An amount of water added to the analytical sample will wet it sufficiently to maximise the extraction efficiency [SANCO, 2013]. As a consequence of this matrix effect, the PT should take this into account when participants report their results. The FAPAS method questions incorporate this additional information for cereals which isn't asked for in other matrix types.

There is insufficient data for a thorough examination of the effect of wetting in the PT data (the large majority of participants do report that they wet the sample first). A brief examination of the data for FAPAS PT 0995 (wheat flour containing deltamethrin, dimoxystrobin, fenamiphos sulfoxide, fenvalerate, isofenfos-methyl, tebuconazole) [FAPAS Reports] shows that there is a greater extraction efficiency from a wet sample by approximately 8% (data not shown).

Involuntary contamination

Many food analyses are concerned with detecting contamination of food items with components that should not be present. The associated PTs therefore attempt to replicate this by supplying a deliberately contaminated test sample. This works well for PTs in which there is one sample to quantitatively analyse the target contaminants. A complication can arise in detection PTs (qualitatively determining presence/absence) in which one sample is deliberately contaminated and a second one is not. The complication takes the form of unintended contamination of the base matrix, which is otherwise deemed to be 'blank'. This incurred contamination is to be avoided if possible but what better challenge is there to have not only a real food matrix but one that is genuinely contaminated? The challenge for the PT provider is to characterise that material, made more difficult where low level contamination is only detected by the most sensitive methods.

Two examples of unintentional contamination are in the fields of allergen and genetically modified (GM) materials testing. The problem of allergens contamination is well documented, certainly in the EU as far as labelling legislation [EU, 2012] is concerned. Many food producers demonstrate awareness of the problem with labelling that refers to the product being handled in a factory that also handles allergenic ingredients. It should come as no surprise, therefore, that sourcing uncontaminated ('blank') base materials for PT purposes is difficult.

■ GM materials

This problem of contaminated matrices is most starkly demonstrated in GM PT. Controls can be put in place in factories that handle food products but controls are less effective in the outside environment. This becomes evident when participants in a PT report the detection of a GM event that wasn't intended to be in the test sample.

A typical mixed flour GM PT will comprise a base matrix of organically-sourced soya, wheat and maize flours, into which have been spiked a number of GM flours (soya and/or maize). The GM flours are sourced directly from the original producer, so their provenance is known. Participants in the PT will be asked to detect and/or quantify genetic elements and specific GM events from a target list and their results reported together with their limit of detection (LOD) or limit of quantification.

Table 3a lists the summary of results from PT MU39 (April 2015) [FAPAS Reports]. The test material was spiked with GA21, MIR604 and MON89034 maize. No GM soya was used in the preparation of the test material. The qualitative results are typical of this type of PT, in that all participants have correctly detected GA21, MIR604 and MON89034 maize in the test material. The quantitative data from this PT are also sufficiently robust (not always the case for GM) that z-scores could also be issued to those participants that submitted quantitative data.

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The genetic elements p35S and tNOS were also detected by participants but this is not unexpected, since the spiked GM events would contain these elements.

TABLE 3a / Summary of qualitative results from PT GeMMA MU39, mixed flours.

Genetic element or event	Result	Agreement (%)
p35S	Positive	100
tNOS	Positive	100
Roundup Ready® soya	Positive	72
MON89788 soya	Negative	100
Bt176 maize	Negative	100
Bt11 maize	Negative	100
MON810 maize	Negative	100
GA21 maize	Positive	100
NK603 maize	Negative	100
TC1507 maize	Negative	100
MON863 maize	Negative	95
MIR604 maize	Positive	100
MON88017 maize	Negative	94
MON89034 maize	Positive	100

The result that stands out is that of Roundup Ready® soya (40-3-2) (RRS®), which 72% of participants have detected despite it not being deliberately used in the preparation of the test material. This is where the reported LOD by participants have in the past indicated a dependency of low LOD with positive detection. With more participants now reporting lower LODs (in the region of 0.01%), this dependency with positive detection is less clear. To illustrate this, Table 3b lists the first six laboratory results for RRS®. Two laboratories have reported an LOD of 0.01% and two have reported an LOD of about 0.05%. For both LOD levels, one laboratory has detected and one has not detected RRS®. These results highlight the low level endemic contamination of the environment with some GM materials but also the advantage of receiving a PT sample that truly reflects sampling circumstances.

TABLE 3b / First six laboratory results for RRS® (40-3-2) from PT GeMMA MU39, mixed flours.

Laboratory number	Result	LOD (%)
001	Detected	*
002	*	*
003	Detected	0.01
004	Detected	<0.045
005	Not detected	<0.05
006	Not detected	0.01

* no data entered

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■ Incurred chemical residues

The issue of GM contamination above tends to be limited to very low levels (probably <0.1%) for analytes which normally are being determined at about 1%. Higher level contamination (at levels normally being monitored) can be used to advantage in a PT. Several examples could be used to illustrate this, including veterinary drugs, mycotoxins and pesticides residues. The distinction between veterinary drug residues and the other chemical contaminants is that the former are largely banned substances in the human food chain whereas the others have tolerated limits. This in turn dictates how an incurred PT material can be produced to accurately mimic real matrices. Bulk materials for mycotoxins or pesticide residues can be screened for the possible presence of incurred residues whereas an animal has to be deliberately dosed to obtain an incurred veterinary drug.

In the case of veterinary drug residue test materials, a residue incurred through a dosing study serves two additional purposes for the PT. First, it will have metabolised to a certain degree and, second, it will be more tissue-bound than a drug spiked onto the matrix. Where the metabolites form part of the overall residue definition, this has obvious consequences for the overall drug residue determination. Most drug residue monitoring schemes will be determining tissue samples from animals that may have been dosed in uncontrolled circumstances. Hence, tissue-bound residues need to be determined using methods that apply appropriate extraction steps. An incurred drug residue PT sample will most closely resemble the type of samples actually received routinely by the participating laboratory.

An incurred drug residue PT adds complexity to the PT, not just in terms of the sample preparation but also with respect to the performance assessments. The PT provider can distinguish between results for the bound drug and results for the total (bound plus free) and highlight where improvements need to be made, especially for determining just the bound drug. Table 4 illustrates this with the summary results for PT 02240 [FAPAS Reports], the nitrofuran metabolite AMOZ in chicken muscle prepared from a dosing study. Forty participants reported results for the total AMOZ but just under half (19 participants) reported for the bound AMOZ. A further consequence of the bound AMOZ results was that the uncertainty of the consensus assigned value was high, reflecting the added difficulty of quantifying the bound drug.

TABLE 4 / FAPAS PT 02240 summary of results, nitrofuran metabolite AMOZ in chicken muscle.

Analyte	Assigned value µg/kg	Number of scores $\|z\| \leq 2$	Total number of scores	% $\|z\| \leq 2$
AMOZ (bound)	*0.99**	*10**	19	*53**
AMOZ (total)	2.16	35	40	88

* data issued for information only

Summary

A proficiency test sample ideally should resemble a real-life sample that would be received by the participating laboratory. This will not be an exact replica, since the PT test items must be homogeneous across all participants. Hence, a puree of lettuce, for example, will be the test sample, rather than a whole head of lettuce. However, there are further implications for the test samples in that method dependency will dictate critical parameters. The critical method parameters might be applied before the analysis begins (instructions to participants) or after

the PT closes (evaluation by the PT provider of data received). In reality, there is likely to be a combination of instructions to consider which allude to how the data will be assessed once all the participants' results are in. This is good practice for a PT provider in any case, as well as a requirement of the standard [ISO/IEC 17043:2010]. The provision of such instructions before the test begins means that they need not be followed exactly by participants but will indicate the effect on assessments if they are not followed.

In the case of the total fat hydrolysis and microbiology preparation instructions (detailed above), this serves dual purposes. It demonstrates how assessments will be affected and it provides equivalence to real food samples.

Capturing participants' method details is clearly desirable from the point of view of assessing results against critical method parameters. However, the example provided above additionally demonstrates the complexity of methods that exist in food testing laboratories. These complexities might have derived from necessity (availability of equipment or materials) or from the variety of food matrices that laboratories actually receive routinely. The PT samples which emulate the food matrices address the reality of the situation.

The production of a PT material and its characterisation is time consuming but essential to provide a realistic test item. A contaminated or incurred material (either intentional or not) can be utilised by the PT provider to advantageous effect. The examples above of GM and veterinary drug residues discuss this. This serves to highlight not just the performance of the participating laboratories but also to provide a true interlaboratory capability comparison.

Acknowledgements

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TABLES 2 / Participants' method information from FAPAS PT 25131, nutritional components in canned fish paste.

n = number of laboratories responding to this question

Moisture	n		n
Is the method used accredited?		**Apparatus used for constant weight**	
Yes	28	conventional oven	25
No	4	microwave oven	1
		vacuum oven	1
		oven forced air circulation	1
What is your method based on?		**Oven temperature (°C) for moisture determination**	
International Standard	8	<100	23
National Standard	13	≥100 - <105	21
In-house method	5	≥105 - <110	6
		≥110 - <150	2
Sample weight (g)		**Time heated for moisture determination (hours)**	
≥1 - <2	2	<1	1
≥2 - <5	13	≥1 - <5	13
≥5 - <10	15	≥5 - <10	11
≥10 - <25	2	≥10 - <24	5
		≥24 - <36	2
Moisture determination procedure		**Desiccator used to cool sample?**	
to constant weight	27	yes	31
4 h at 103°C	1	no	1

Ash	n		n
Is the method used accredited?		**Ash furnace temperature (°C)**	
Yes	26	≥500 - <550	11
No	3	≥550 - <600	17
		≥600 - <650	1
What is your method based on?		**Time in ash furnace (hours)**	
International Standard	7	<1	1
National Standard	12	≥1 - <5	6
Manufacturer/Kit instructions/Technical note	1	≥5 - <10	8
In-house method	5	≥10 - <15	5
		≥15 - <24	6
		≥24 - <48	2
Sample weight (g)			
≥1 - <2	4		
≥2 - <5	14		
≥5 - <10	10		
≥10 - <25	1		
Steps taken to avoid spattering			

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Ash	n		n
charred on hotplate before ashing	8		
crucible with lid	2		
pre-dried in oven	11		
pre-dried on steam bath	2		
slow temperature ramp	7		
charred on Bunsen burner	1		
none	1		

Total fat	n		n
Is the method used accredited?		**Pre-extraction stage?**	
Yes	27	Yes	7
No	4	No	24
What is your method based on?		**Pre-extraction time (hours)**	
International Standard	7	<1	6
National Standard	12	≥1 - <2	3
Paper Published in an international journal	1	≥2 - <3	3
Manufacturer/Kit instructions/Technical note	1	≥3 - <4	1
In-house method	8		
Sample weight (g)		**Extraction solvent components**	
≥1 - <2	7	diethyl ether	7
≥2 - <5	15	hexane	2
≥5 - <10	8	petroleum ether/spirit	20
≥10 - <25	1	chloroform: methanol (2:1)	1
		diethyl ether - petroleum ether	1
		none	1
Acid/alkaline hydrolysis used?		**Total extraction time (hours)**	
acid	20	<1	8
alkaline	1	≥1 - <2	8
none	10	≥2 - <5	9
		≥5 - <10	2
		>10	2
Total fat extraction method			
CEM	3		
Mojonnier	5		
SBR	1		
Soxhlet	9		
Soxtherm	1		
Tecator/Soxtec	4		
Weibull-Stoldt	3		
Acid hydrolysis	1		
acid hydrolysis +n-hexane extraction	1		
Folch et. al	1		
NMR	1		

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Nitrogen	n		n
Is the method used accredited?		**Digestion acid**	
Yes	18	hydrogen peroxide	2
No	1	sulphuric acid	26
What is your method based on?		**Catalyst**	
International Standard	6	copper (Cu)	17
National Standard	10	potassium (K)	8
Manufacturer/Kit instructions/Technical note	1	mercury (Hg)	2
In-house method	7	selenium (Se)	4
		titanium (Ti)	2
		3.5 g K2SO4 + 3.5 mg Se	1
		copper sulphate	1
		Kjeltab	1
Sample weight (g)			
<1	11		
≥1 - <2	15		
≥2 - <5	4		
Determination method			
Dumas	3		
Kjeldahl	18		
Kjeltec-Tecator System	7		
LECO	1		
Super Kjel	1		

Sodium	n		n
Is the method used accredited?		**Digestion**	
Yes	20	dissolve in acid	15
No	5	dissolve in water	2
		microwave digestion	6
What is your method based on?		**Pre-treatment**	
International Standard	7	caesium addition	3
National Standard	5	dissolution	2
Manufacturer/Kit instructions/Technical note	2	filtration	1
In-house method	4	heat	2
		heating with HCl	3
		internal standard addition	1
Sample weight (g)		**Determination**	
<1	5	cold vapour / hydride generation AAS	1
≥1 - <2	7	flame AAS	6
≥2 - <5	7	Flame Photometry	5
≥5 - <10	4	ICP	1
≥10 - <25	2	ICP-MS	4
		ICP-OES	5
		By calculation from sodium chloride	1

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Sodium	n		n
Sample preparation			
dry ashing	12		
wet ash	3		
acid digestion	1		
digestion	1		
digestion with nitric acid	1		
dissolved in water, filtration	1		
wet digest	1		

Chloride	n		n
Is the method used accredited?		**Time in ash furnace (hours)**	
Yes	17	≥1 - <5	1
No	9	≥5 - <10	2
		≥10 - <15	2
		≥15 - <24	2
What is your method based on?		**Steps taken to avoid spattering**	
International Standard	9	charred on hotplate before ashing	2
National Standard	7	crucible with lid	1
Manufacturer/Kit instructions/Technical note	1	pre-dried in oven	5
In-house method	3	slow temperature ramp	2
Sample weight (g)		**Sample preparation**	
≥1 - <2	4	cold water extraction	7
≥2 - <5	11	hot water extraction	4
≥5 - <10	7	nitric acid added	5
≥10 - <25	2	water added	6
		ammonia extraction	1
Was sample ashed?		**Determination**	
Yes	7	argentometric titration	7
No	17	chloride analyser	1
		Mohr	3
		potentiometric method	3
		thiocyanate titration	5
		Volhard	5
		ICP-MS	1
Ash furnace temperature (°C)			
<500	1		
≥500 - <550	4		
≥550 - <600	2		

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The provision of proficiency testing for TSE rapid tests by the APHA

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Abstract

Under Regulation (EC) No. 999/2001, the Animal and Plant Health Agency (APHA) in the UK, in its capacity as a European Union Reference Laboratory (EURL), is responsible for the provision of an annual programme of proficiency testing (PT) schemes for the assessment of diagnostic procedures for transmissible spongiform encephalopathies (TSEs) at both European and national levels. These ISO17043-certified schemes target European Union (EU)-approved commercial screening tests used to carry out surveillance of bovine spongiform encephalopathy (BSE) and scrapie. Reference materials are generated from whole tissues; all include the marker protein PrPres in a range of concentrations similar to those found in both clinical and preclinical animals. Participating laboratories must perform satisfactorily in these PT schemes to be authorised to carry out national surveillance testing. Root cause analysis indicates that PT failures are most likely to occur as a result of inadequate laboratory protocols or training. However, trend analysis of EU National Reference Laboratory (NRL) test data demonstrates that, despite variation in the quantitative performance of tests, standards are being maintained at a high level despite a declining prevalence in disease across Europe.

Keywords

- BSE
- Laboratory proficiency testing
- Rapid test
- Scrapie
- Statutory

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Introduction

Transmissible spongiform encephalopathies (TSEs), such as bovine spongiform encephalopathy (BSE) in cattle and scrapie in small ruminants, are a group of rare neurodegenerative disorders affecting the central nervous system of both humans and animals that can arise as genetic disorders, transmit directly from animal to animal or be transmitted through the presence of infectivity in feed or food [Mastrianni, 2010; Bruce *et al.*, 1997; Wilesmith *et al.*, 1988]. These disorders are also referred to as Prion diseases and are characterised by the accumulation of an abnormal isoform of a host-encoded protein called prion protein (PrP) [Collinge and Clarke, 2007]. This abnormal prion protein (PrPres) is disease-specific, protease-resistant and is currently the most consistent marker for disease. PrPres can only be reliably detected on post-mortem examination.

In order to safeguard public and animal health, the European Commission introduced Regulation (EC) No. 999/2001 laying down rules for the prevention, control and eradication of certain TSEs [EU, 2001], and each Member State was required to nominate a National Reference Laboratory (NRL) to implement the requirements of the regulation. An over-arching EU reference laboratory (EURL) was also nominated to oversee testing and related activities. First published in 2001, and updated on numerous occasions in line with updated scientific evidence, these regulations define the functions and responsibilities of the TSE EURL and each individual Member State NRL, along with rules for sampling and laboratory testing approaches. The Animal and Plant Health Agency (APHA) has been the UK NRL and the EURL since 2001.

Due to the effect of control policies introduced at a European level, the prevalence of naturally occurring cases of TSE in food animal species has declined. However, the statutory testing defined in the Regulation means that over 3 million cattle and almost 500,000 small ruminants were subjected to screening for TSEs in 2013 [EU, 2015].

TABLE 1 / TSE rapid tests approved for use in Europe and defined within Commission Regulation (EU) No 1148/2014 [EU, 2014].

Rapid Tests	BSE Monitoring	Scrapie Monitoring
Bio-Rad TeSeE® SAP	✓	✓
Bio-Rad TeSeE® Sheep/Goat	×	✓
IDEXX HerdChek® BSE-Scrapie Antigen EIA	✓	✓
Prionics®-Check LIA	✓	×
Prionics®-Check Western	✓	×
Prionics®-Check PrioSTRIP	✓	×
Prionics®-Check PrioSTRIP SR, visual reading protocol	×	✓
AJ Roboscreen BetaPrion® BSE EIA	✓	×

The EU legislation covering TSEs clearly defines the commercial screening tests that have been approved by the Commission for use in the statutory surveillance of TSEs (Table 1). Prior to approval, these tests were subject to independent evaluation of test performance for specific forms of TSE [EFSA, 2009; EFSA, 2012]. Commonly known as 'rapid tests', they all use the principle of immunocapture to detect the presence of PrPres. The test platforms include an immunoblot and a variety of different immunoassays, including sandwich, microplate-based, chemical polymer and lateral flow immunoassays. The common feature of all of these tests is that they enable high throughput testing which is fast and reliable.

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Overview of TSE proficiency testing

Proficiency testing (PT) allows samples of known but undisclosed provenance to be introduced into laboratories for the purpose of providing independent and confidential verification of the testing process. This is essential for assuring the European Commission, the EURL, NRLs and other testing laboratories of the continued competence of laboratory staff and ensuring the maintenance of technical expertise.

Under the TSE legislation, the APHA has responsibilities under both its TSE EURL and NRL remit to provide periodic PT to assess diagnostic procedures at the EU and national levels. This service is also provided commercially to TSE screening laboratories worldwide.

All EURL PT testing for TSE rapid tests in cattle and small ruminants is distributed through the APHA Quality Assurance Unit 'Vetqas', an internationally recognised market leader in the provision of PT schemes for veterinary laboratories that currently offers a range of veterinary schemes (http://ahvla.defra.gov.uk/apha-scientific/services/vetqas/index.htm).

■ Reference materials

Prion protein cannot be propagated in vitro in quantities sufficient for use in PT schemes. Reference material therefore has to be created from whole tissues from a variety of sources. Where possible, these are natural field cases of TSEs, identified through both active and passive surveillance routes in the UK. Additionally, the TSE EURL receives funding directly from the EC to generate infected tissue from animals experimentally inoculated with less prevalent strains of TSE, which cannot be sourced in sufficient quantity (if at all) from natural cases. These experimental tissues are generated specifically for use in PT schemes and test evaluation exercises.

All source materials used in the manufacture of TSE reference samples are managed by the APHA TSE Biological Archive, the largest and most comprehensive repository of TSE material worldwide (http://www.tse-lab-net.eu/biological-archive/index.html).

■ Preparation and storage of PT samples

All samples distributed within TSE rapid test PT schemes are brain homogenates or macerates which have been prepared at APHA using protocols developed by the TSE EURL so that they are suitable for use by all EU-approved rapid tests. All tissues are initially cleaned to remove connective tissue and other debris. To prepare tissue homogenates, samples are combined with an equal volume of purified water and then homogenised using a household hand blender for three bursts of 30 seconds. The addition of purified water ensures the creation of a homogeneous product which can be easily diluted using negative tissue homogenates from the same species in order to provide a range of PT samples (classified as strong, medium or weak positive) to mimic the range of PrPres signals that may be expected in preclinical animals [Arnold *et al.*, 2007]. The only exception to the above protocol is for atypical scrapie samples, which are finely diced with no addition of purified water, resulting in a fine macerate. This approach was developed following anecdotal data from NRLs, and EURL observations that atypical scrapie PrPres signal stability could be compromised by sample homogenisation.

Homogenates and macerates are generally subdivided into aliquots of approximately 1.3 g. This provides sufficient material for each participant to carry out up to three different tests, depending on the methods in use within their laboratory.

Prior to their use as PT samples, 4% of the total number of aliquots from each batch are pre-tested by an EU-approved rapid test, and a single sample is tested by Western blot. This establishes both consistency across aliquots (as required by ISO/IEC 17043 Conformity As-

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sessment – Guidelines for Proficiency Testing) and a baseline value (the intended result) for assessing samples as strong, medium or weak positive.

Following preparation and initial suitability testing, samples are returned to dedicated PT sample storage at -80°C until required for use. A specialised database is used to trace the history, storage location and destination of each sample used in PT distributions.

■ TSE PT Schemes

APHA currently provides ISO/IEC 17043-accredited PT schemes for both bovine and small ruminant rapid tests. TSE testing laboratories working to ISO/IEC 17025 or equivalent (as required by EC Regulation 882/2004), can use successful participation in these schemes as evidence of competence for third party accreditation bodies.

The TSE EURL currently holds multiple aliquots from 17 bovine and 27 small ruminant sources representing all currently identified TSE field strains. This allows the delivery of targeted rounds, which may include all the TSE types known to infect each species. These schemes are distributed 1-3 times per year and comprise a combination of 5 to 7 positive or negative samples (Table 2). The positive samples are selected to represent a variety of target signal intensity, including those signal intensities that may challenge the detection threshold of approved rapid tests. Inclusion of samples from the same batch in multiple PT schemes enables monitoring of signal stability over time. In addition, duplicate samples may be included within a single distribution to further challenge test repeatability.

TABLE 2 / Summary of annual TSE rapid test PT schemes organised by APHA in 2015.

PT Distribution type	Target	Frequency (per year)	No of participating laboratories (2015)	No of samples in distribution
Bovine BSE Rapid Test	EU NRLs	1	26	7
	UK Diagnostic Laboratories	3	2	7
	Worldwide Commercial	2	9	5
Small Ruminant Scrapie Rapid Test	EU NRLs	1	27	7
	UK Diagnostic Laboratories	2	2	7
	Worldwide Commercial	2	8	5

Each PT distribution is dispatched in a frozen state as UN3373 Biological Substance Category B. Samples are blind labelled and provide enough tissue for 2-3 different tests to be carried out, enabling sufficient available material for testing labs to maintain proficiency using a number of different test methods or personnel, if required.

■ Reporting and analysis of results

Vetqas operates an on-line Proficiency Testing Laboratory Information Management System (PT LIMS). Participating laboratories are allocated a confidential laboratory ID number which

they use when entering their test results and supplementary supporting information such as test expiry dates, positive and negative control values and test cut-offs directly onto the PT LIMS.

Participants are encouraged to provide comments and raw data in support of their reported results. Such supporting information also provides assurance that laboratories are able to correctly interpret their testing outcomes and would take the correct action if an anomalous result occurred under routine testing conditions.

Once participants have entered their results, PT LIMS is used to produce tabulated reports which are reviewed and commented upon by the EURL. Reports receive a conformity check by Vetqas staff, and an e-mail is then sent from PT LIMS informing participants that tabulated results and commentary are available to view.

■ Follow-up of anomalous results

If any laboratory performing statutory testing fails to demonstrate their competence in EURL PT schemes, the Commission requires the EURL to suspend the testing activities of that laboratory. Such laboratories must submit an anomaly report to the EURL and refer testing activities to a competent laboratory until issues have been resolved.

In discussion with the affected laboratory, the EURL performs a root cause analysis based upon scrutiny of the original raw data and the submitted anomaly report. Root cause analysis has identified several reasons why participants fail to provide the expected diagnostic result. These have included transcription error, operator error, equipment or test kit failure, accidental or deliberate use of out of date kits, failure to follow instructions as stated within test kit inserts, possible sample misidentification, variations in signal stability across different combinations of TSE strains, and test kit format.

If the error identified through root cause analysis is not technically based, *e.g.* transcription error, and the laboratory has demonstrated that protocols have been reviewed and retraining has taken place, then the EURL can elect to allow the laboratory to resume testing without undertaking any follow-up PT.

When technical issues are identified, the laboratory must confirm that the appropriate corrective actions and retraining have been completed. Competence must then be demonstrated by successful participation in a follow-up PT scheme before statutory testing can be resumed.

Trend Analysis of PT data

Regardless of the test being used, variability at a laboratory level may lead to differences in absolute test outputs. The data in figures 1, 2 and 3 illustrate a single representative PT distribution for three different bovine rapid tests. Each set of data was generated from laboratories that used the same test batch. It can be seen that, despite the differences between absolute values generated by laboratories, performance is generally consistent within any individual laboratory when individual replicate samples are compared, or where laboratories have carried out tests twice.

The ability to differentiate between strong, medium and weak positive samples varies depending on the test used. The absolute values generated by tests 1 and 3 reflect the intended sample strength (strong, medium and weak positive), however they differ in the range of data submitted, for example the range of data submitted for sample 1 using test one was 0.437-3.176 (upper limit of detection 3.5) and the range for test 3 for the same sample was

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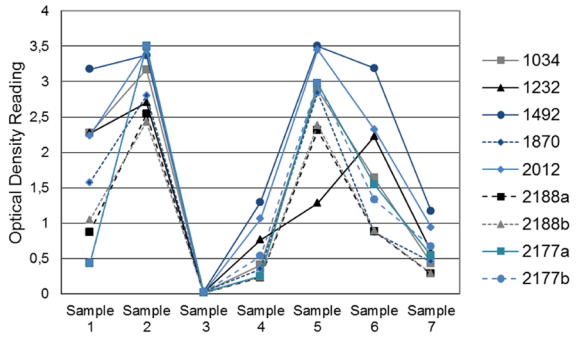
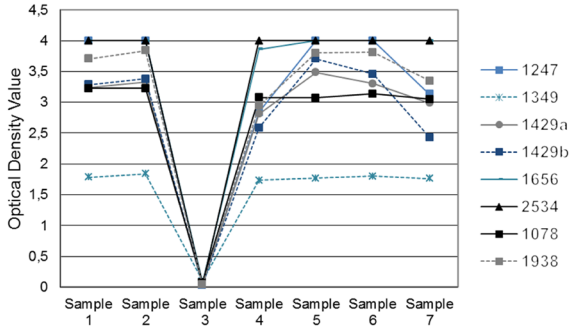
FIGURE 1 / Bovine rapid test 1 data from all laboratories using the same batch of a single test.



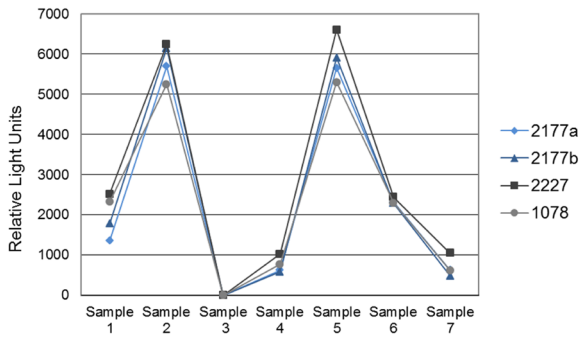
Each coloured line represents a single laboratory identified by its confidential laboratory ID number. Laboratories carrying out the tests twice are denoted by a or b. Samples 1 and 6 are medium positive replicates, samples 2 and 5 are strong positive replicates, samples 4 and 7 are weak positive replicates.

FIGURE 2 / Bovine rapid test 2 data from all laboratories using the same batch of a single test.



Each coloured line represents a single laboratory identified by its confidential laboratory ID number. Laboratories carrying out the tests twice are denoted by a or b. Samples 1 and 6 are medium positive replicates, samples 2 and 5 are strong positive replicates, samples 4 and 7 are weak positive replicates.

FIGURE 3 / Bovine rapid test 3 data from all laboratories using the same batch of a single test



Each coloured line represents a single laboratory identified by its confidential laboratory ID numberLaboratories carrying out the tests twice are denoted by a or b. Samples 1 and 6 are medium positive replicates, samples 2 and 5 are strong positive replicates, samples 4 and 7 are weak positive replicates.

1356-2519 (upper limit of detection >6,600), a much tighter distribution. Conversely, data from test 2 indicates that this sample panel does not appear to fully challenge the lower limit of detection threshold for this test.

However, the basic requirement of all screening tests is that they are able to distinguish between positive and negative samples under field conditions. EURL analysis of PT data clearly shows that the laboratories currently undertaking TSE surveillance testing in the EU are doing so competently, with only 0.224% (6 in 2675) PT test failures occurring between 2009 and 2015, attributable to operator errors.

Root cause analyses have shown that a small number of infrequent anomalies relate to human factors, such as sample handling errors. Consequently, the EURL is of the opinion that the key to sustaining test competency lies in the maintenance of effective laboratory protocols and operator training.

Despite the variation observed, the sample panels used are appropriate for assessing the performance of the tests at the qualitative level, which is the primary requirement of these exercises. Additionally, examination at a quantitative level helps the EURL to informally monitor test performance, both in terms of batch quality and of individual laboratory performance. Batch performance can be monitored at a single time point using data generated from within a single distribution, or within the same laboratories at multiple time points using data gathered from multiple distributions, allowing the EURL to raise any emerging issues with the manufacturers for investigation. Issues relating to dips or increased variability in individual laboratory performance may also be identified using single sample data gained at single/multiple time points from single/multiple distributions that can be investigated at a local level.

These data are therefore of value in the ongoing assessment of both test and laboratory performance, providing European laboratories with confidence in their ability to successfully maintain these tests in spite of the declining prevalence of TSE disease and reducing surveillance requirements.

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Conclusion

TSE rapid screening tests are designed to differentiate between positive and negative samples. Through the provision of periodic proficiency testing using sample panels which are suitable for use on all EU-approved rapid tests, the TSE EURL and NRL assess the diagnostic capability of laboratories at both EU and national levels. Overall, the test data generated within these PT schemes supports the robustness of the tests currently in use.

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How to do more with limited resources: the example of a shared quality management system for the organisation of inter-laboratory proficiency tests in the French Plant Health Laboratory

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Abstract

As French National Reference Laboratory for the detection and identification of plant pests, the ANSES Plant Health Laboratory organises proficiency tests in order to ensure that laboratories certified by relevant authorities are capable of producing reliable analytical results.

Since 2014, the Plant Health Laboratory—which is composed of several separate technical units—has developed a centralised quality management system for organising proficiency tests within its different disciplines.

This paper presents the specificities of this management system based on in-house subcontracting and the strategy adopted by the Plant Health Laboratory to meet the requirements of ISO/IEC 17043.

Keywords

- Detection
- Identification
- ISO 17043
- Laboratory proficiency testing
- Plant pests
- Quality management

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Introduction

According to European regulation (EC) No. 882/2004 [EU, 2014], reference laboratories have to supervise and coordinate the activities conducted by certified laboratories (*i.e.* officially certified by a relevant authority) in order to ensure that they are capable of producing reliable analytical results. Proficiency testing (PT) is a way of checking laboratory testing performance by means of an inter-laboratory comparison. Non-compliant results in proficiency testing can have important consequences for the laboratory, such as the suspension or withdrawal of its official certification and/or its accreditation. It is therefore essential that the PT organiser be able to provide participants with a guarantee of its competence in organising proficiency tests.

ANSES, the French Agency for Food, Environmental and Occupational Health & Safety, has 11 reference and research laboratories, including the Plant Health Laboratory (PHL). The latter is the French National Reference Laboratory for the detection and identification of plant pests. In this context, it is involved in designing and organising inter-laboratory proficiency tests in which certified French laboratories are required to take part.

In 2013, the PHL's Unit for Tropical Pests and Diseases, based on Réunion Island, was accredited by French accreditation body COFRAC to organise plant health proficiency tests. This accreditation was formal recognition of its competence in organising proficiency tests and reliably assessing participants' proficiency.

This experience was the foundation on which a centralised quality management system was developed for all the PHL's units and disciplines. In this context, the proficiency testing service (PT service) was developed within the PHL to organise proficiency tests for detecting and identifying plant pests in accordance with the general requirements of standard ISO/IEC 17043 [2010], which is the normative reference for the organisation of inter-laboratory proficiency tests.

Proficiency tests in the area of plant pests

The usual procedure for proficiency tests to detect and/or identify plant pests entails an organising laboratory (the organiser or provider) sending identical sets of samples to a group of participating laboratories (the participants) for the detection/identification of one (or more) target plant pest(s). The samples are intended to simulate the kind of samples that are routinely analysed. The participants are not informed of the expected results (assigned values), and are requested to perform the (blind) analyses just as for routine samples. To ensure that the inter-laboratory comparison is reliable, samples are validated by the organiser in terms of status (the assigned value results from the organiser's own experimentation and is consequently defined independently from the participants' results), homogeneity and stability. The results obtained from the samples are returned to the organiser to assess compliance with the expected results. A PT report containing the results of this performance assessment is drafted and sent to each participant.

Good results provide independent and objective evidence of effective analytical quality assurance [Stuart and Squirell, 2001], and encourage the laboratory to maintain this high level of performance. Conversely, poor results can help reveal anomalies in the analytical process. Subsequently, a causal analysis is conducted and an action plan implemented to improve the laboratory's performance.

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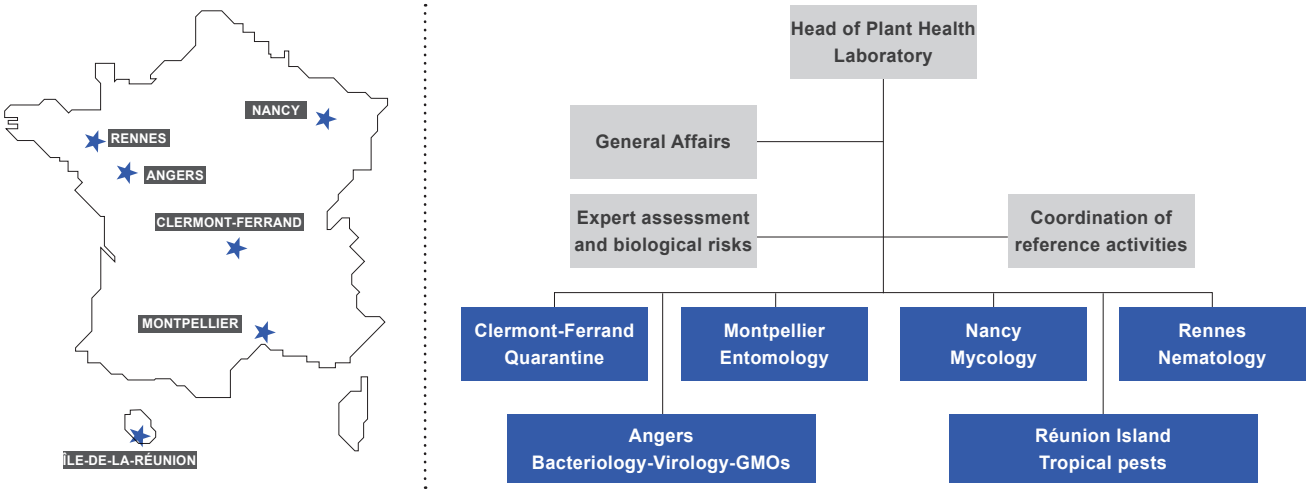
Organising plant health PT within ANSES

■ In-house subcontracting

The Plant Health Laboratory is composed of six specialised sites (Figure 1): Angers (bacteriology, virology and genetically-modified organisms), Clermont-Ferrand (virology and quarantine facilities), Montpellier (entomology), Nancy (mycology), Rennes (nematology) and Saint-Pierre on Réunion Island (tropical pathogens). The administrative headquarters are located in Angers, as are three cross-functional units which organise and coordinate the activities of the six specialised technical units.

FIGURE 1 / Organisation of the Anses Plant Health Laboratory (with specialised technical units in blue).



The proficiency testing service, which is part of the cross-functional «coordination of reference activities» unit, is in charge of organising proficiency testing (PT) on behalf of the PHL. To do so, the PT service relies on the PHL's specialised technical units within the framework of an in-house subcontract (support agreement). This subcontract concerns the technical activities of preparing, validating, packaging, labelling, and shipping the samples. The PT service does not subcontract the planning of the proficiency test scheme, evaluation of performance or authorisation of the final report, as shown in figure 2.

The subcontracted work is covered by a support agreement between the PT service and each specialised technical unit, with general provisions applicable to all proficiency tests organised under the support agreement. The support agreement provides the overall framework. The specific provisions applicable to each proficiency test are described in the PT plan established prior to the proficiency test and signed by both the PT service and the specialised technical unit. The PT service remains responsible for the subcontracted work with respect to participants and other interested parties.

■ Organisation of the PT service

Organising proficiency tests involves different key functions within the PT service:

- the head of the PT service, responsible for its management in conjunction with the head of the PHL, authorises distribution of the PT reports;
- test coordinators. There is one coordinator per discipline or type of proficiency test: one coordinator for the nematology proficiency tests (working in the Rennes technical unit), two coordinators for the virology proficiency tests (one in the Angers unit and the other

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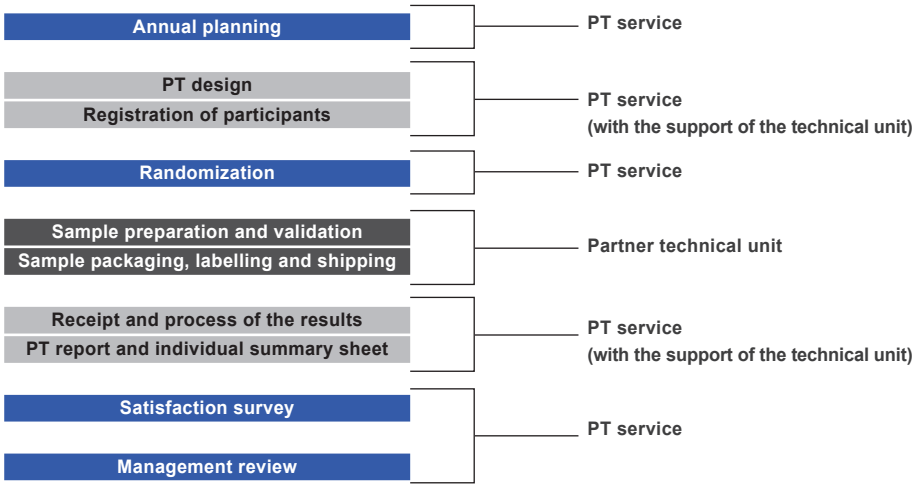
in Clermont-Ferrand, each with different areas of competence), one for the bacteriology proficiency tests (also working within the Angers unit), one for the mycology proficiency tests (working in the Nancy unit), one for the entomology proficiency tests (in the Montpellier unit) and finally one for the proficiency tests in tropical bacteria and viruses (in the Réunion island technical unit). The coordinators are the project leaders for the proficiency tests within their area of competence: they each have to coordinate the test design and oversee all work and activities relating to its implementation (whether technical operations, statistical analysis, etc.). They guarantee confidentiality and ensure communication with the participants. They also validate the PT report. Each test coordinator works within the technical unit specialising in the discipline concerned, thus facilitating supervision of the work carried out under the support agreement. However, the test coordinators work directly for the PT service and act under its authority.

FIGURE 2 / Roles of the PT service and the supporting technical unit for organising proficiency tests within the PHL.



As the number of coordinators is limited to one per discipline, for this function, the provisions for the appointment of a deputy coordinator require the contribution of a coordinator from a different unit. The deputy coordinator therefore operates remotely.

These provisions, by limiting the number of coordinators, are intended to optimise resources. Thus, the competencies required for this key function are monitored for a limited number of persons. Moreover, each coordinator performs coordination duties more regularly, as either the main or deputy coordinator, thus mastering the tasks in hand. The same proficiency test may be coordinated by the main coordinator, the deputy (who remains in his or her own specialised technical unit) or both together. The deputy coordinator can work remotely thanks to new communication technologies;

- the statistical analyst who helps implement all the statistical operations needed for the design, organisation and interpretation of the proficiency tests;
- the quality staff (quality manager and quality assistant) in charge of the PT service quality management. This includes ensuring that PT is always organised in accordance with the PT service's quality management system.

Organising proficiency tests also involves different key functions within the supporting tech-

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nical unit:

- the head of the technical unit, responsible for its management. The head commits the unit's resources in keeping with its supporting agreement with the PT service;
- the technical manager, responsible for technical operations (*i.e.* selecting appropriate samples then preparing, validating and packaging them);
- technical operators, who conduct the laboratory experiments under the responsibility of the technical manager;
- the operator responsible for labelling who labels the samples and prepares the packages of samples.

Figure 3 shows the PT service's organisation chart and the relations between these different stakeholders.

Although hosted in the PHL headquarters in Angers, the PT service operates on a multi-site basis from day to day. This is possible through information technology tools which foster cooperation through networking, the sharing of knowledge, pooling of information and remote communication/management in real time.

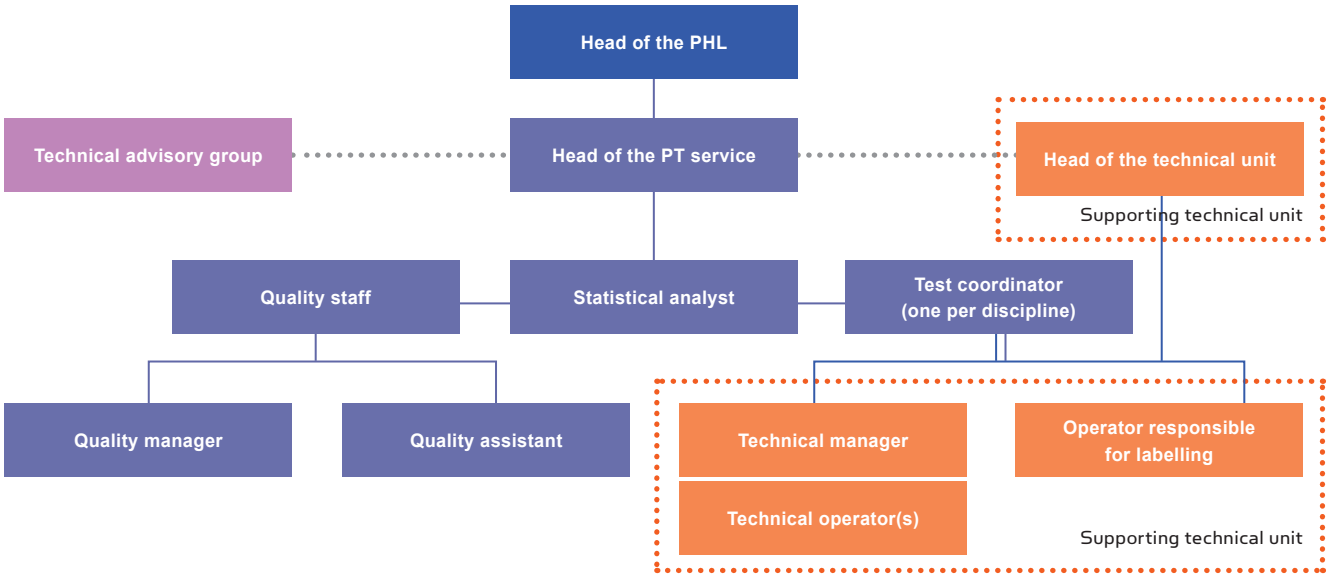
These tools include:

- a shared server designed so that different access rights can be attributed to different users;
- an electronic document management system for the PT service's quality documentation, including its accessibility and use for all users (including supporting technical units);
- computer applications for instant messaging and audio/video/web conferences with screen sharing (*e.g.* Lync);
- computer applications for the multi-site validation of documents while ensuring the security of PDF signatures (*e.g.* Foxit phantom).

■ Management system

Organising proficiency tests is a highly technical activity (Stuart and Squirell, 2001) which must be supported by a solid quality management system and technical skills. The accreditation according to ISO/IEC 17025 [2005] of each supporting technical unit in line with its

FIGURE 3 / Organisation chart of the PT service and relations between the key functions involved in proficiency test organisation.



reference mandate, guarantees both, as ISO/IEC 17025 is the normative reference on the competence of testing laboratories.

The support agreement requires in particular that the technical unit be accredited for the analytical method used to validate the sample, *i.e.* determining the assigned value, and ensuring sample homogeneity and stability.

In addition, to meet the requirements specific to ISO/IEC 17043 [2010], the PT service manages the organisation of each proficiency test as a project based on the PDCA (Plan Do Check Act) continuous improvement model, as shown in figure 4.

When the PDCA approach is applied to each production cycle of a proficiency test, the production steps can be standardised and levers found so that there is continuous improvement from one cycle to the next (virtuous circle).

This improves the overall performance of the PT organisation process. Indeed, the configuration of the PT service, with a single centralised quality management system for several separate technical units, makes it possible to implement corrective/preventive actions cutting across all the disciplines involved. Consequently, it is more efficient in capitalising on information, anticipating risks and ensuring continuous improvement than if each technical unit had its own independent quality management system.

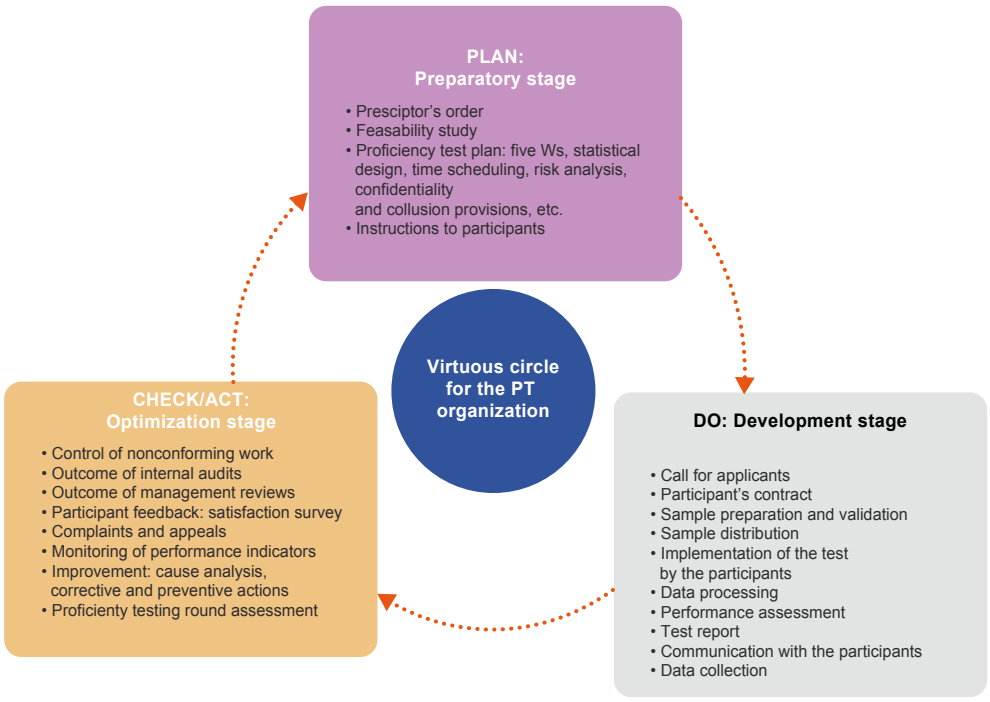
Experience to date

Advantages of this centralised quality management system

There are several advantages to this mutual management system in organising PT. In accordance with ANSES policy objectives, the main advantages are explained hereafter:

- greater legibility for participants: a single documentary system, harmonised practices,

FIGURE 4 / Project management based on a PDCA model applied to the organisation of proficiency tests.



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single centralised management of non-compliances, customer feedback and continuous improvement;

- the rationalisation of resources and means: saving resources (a single quality management systems vs. six potential ones if each technical unit had its own), sharing of tools and information, dematerialisation of documents, and capitalising on the technical units' ISO/IEC 17025 quality management systems;
- better exploitation of information: pooling of non-compliances, generalisation of corrective/preventive actions to all disciplines;
- structural changes (*e.g.* normative change) are easier to integrate;
- greater flexibility and the possibility of a «variable geometry» configuration through in-house subcontracting, so as to offer proficiency tests tailored to the ever-changing phytosanitary context with appropriate responsiveness (possibility of including new technical fields, and resorting to external subcontracting). This is of crucial importance in guaranteeing the import/export of pest-free plant material.

■ Difficulties experienced in this approach

A major difficulty encountered when developing this centralised management system was to obtain the support of all the staff concerned by this project. The change of scale was a key issue to be clarified, so that the technical units did not have the impression that they were losing control. The management team's committed involvement was essential in overcoming these misgivings and creating a climate of confidence.

TABLE 1 / Characteristics of the four PTs organised by the PT service in 2015, completed and with customer feedback.

						Results of the satisfaction surveys		
Proficiency test (N°)	**Field / technique**	**Target organism**	**Number of samples (in each participant panel)**	**Supporting technical unit**	**Number of participants**	**Answer rate**	**Overall rate of satisfaction**	**Detailed rate of satisfaction (each question is considered)**
FD/2015	Virology Real-Time PCR	Grapevine phytoplasmas	22	Angers	9	100%	100%	97%
15PPV	Virology ELISA	Plum Pox Virus	22	Clermont-Ferrand	11	64%	100%	100%
15XD	Bacteriology Conventional PCR and isolation	*Xanthomonas axonopodis* pv. *dieffenbachiae*	16	Réunion Island	7	71%	100%	98%
15BXE	Nematology Real-Time PCR	*Bursaphelenchus xylophilus*	15	Rennes	3	100%	100%	100%
				Total	**30**	**81%**	**100%**	**98%**

More generally, the successful implementation of such a system requires the management team's involvement and rigorous management and monitoring on a daily basis.

■ Customer feedback

Once a proficiency test's report has been issued, participants are invited to take part in a satisfaction survey in the form of an online questionnaire. Clients are also given the opportunity to submit complaints during proficiency testing and, if they do not agree with the performance evaluation, to appeal within one month of the PT report being sent.

No complaints or appeals have been recorded for the four completed proficiency tests orga-

nised by the PT service to date. As detailed in Table 1, the satisfaction surveys covered a total of 30 customers. The response rate is very high (81%). The overall rate of satisfaction is 100% and, in detail (if we consider each question), the rate of satisfaction is 98%. This organisation therefore appears to fully satisfy clients and meet their expectations.

A survey dedicated to the certified French laboratories and more specifically oriented towards this change in organisation will be conducted in mid-2016, when most of these laboratories will have already experienced this new organisation.

Conclusion

The use of analysis methods capable of producing reliable analytical results is a prerequisite to the effective control of quarantine plant pests. Proficiency testing is considered to be one of the most reliable ways of verifying and coordinating laboratories' analytical proficiency.

Plant pest proficiency tests have a number of notable features including the processing of qualitative results [Chabirand *et al.*, 2014; EPPO, 2014]. Not only do laboratories have to demonstrate their ability to produce accurate analytical results, but proficiency test organisers also have to demonstrate their competence in organising proficiency tests within their area of expertise.

The development of this centralised quality management system provides a transition from individual experience to mutual knowledge in the field of PT organisation. This capitalisation of experience has proved valuable not only at the PHL but also more widely. Experts from PHL have notably helped prepare EPPO Standard PM 7/122 [EPPO, 2014].

The centralised quality management system developed by the PHL relies on the strong foundations of the ISO/IEC 17025-accredited systems of the different technical units. Its development was possible because the ISO/IEC 17025 and ISO/IEC 17043 standards share common structure and contents. According to recent exchanges with the French accreditation body, such a centralised quality management system could be accredited provided that there is a close fit between the ISO 17025 scope of accreditation for the technical units and the requested ISO 17043 scope of accreditation for the PHL.

The centralised quality management system developed by the PHL provides an example that could be applied in other fields to entities similarly composed of different "technical units" (*e.g.* food safety or animal health).

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METHOD VALIDATION

Experience in developing generic guidelines for method validation applicable in the areas of animal health, plant health, and food safety

Michel Laurentie*[**].

Abstract

A generic guideline for method validation was developed from all laboratories at the French agency for food, environmental and occupational health and safety (ANSES). This guideline takes into account the specificity of the various subject areas, such as animal health, plant health, or food safety. Furthermore it was developed for both qualitative and quantitative methods. A life cycle for analytical methods was also introduced. The process of validation was described according to the principle that the first step is to determine method performance using standard characteristics (trueness, precision, LOQ, sensitivity, and specificity) for qualitative or quantitative methods, and the second step is to compare the studied characteristics with the validation criteria defined in the tender specifications. This process concludes the validation of the analytical method. Estimating the uncertainty of results was also taken into account.

Keywords

- Guidelines
- Performance characteristics
- Validation criteria
- Validation process
- Uncertainty

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METHOD VALIDATION

Introduction

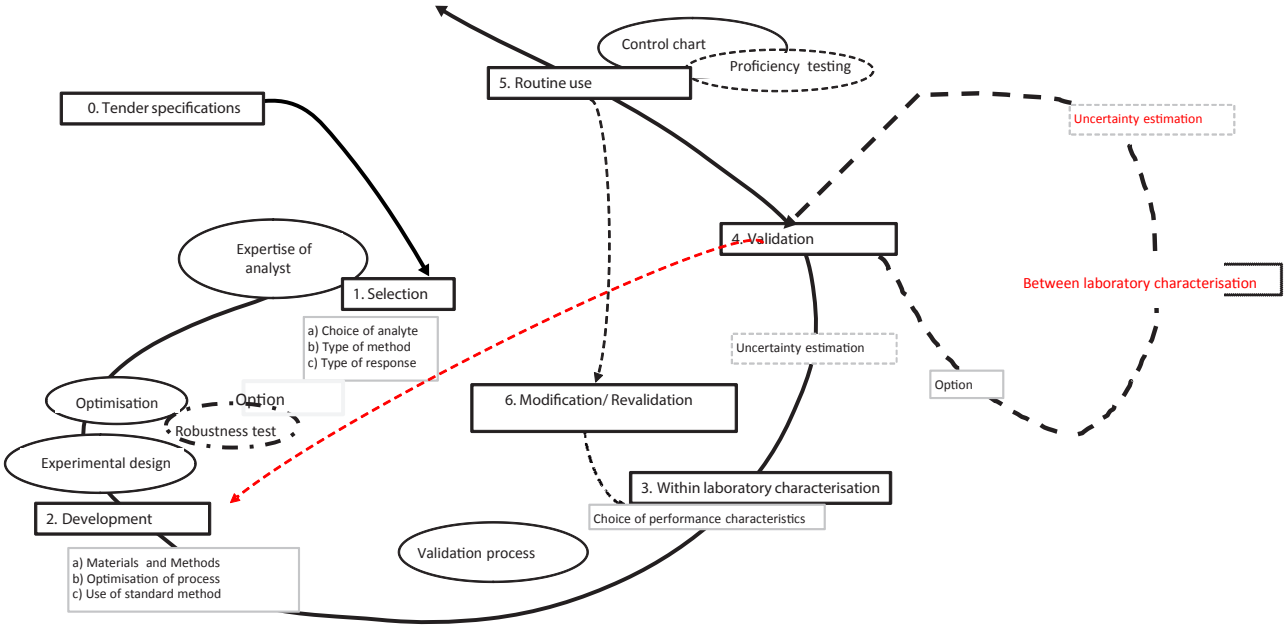
The French agency for food, environmental and occupational health & safety (ANSES) has many laboratories that develop multiple methods in different fields, such as animal health, plant health, and food safety. The majority of methods are in animal health (47%). For food safety and plant health, the percentages are 32% and 20%, respectively. Most of these laboratories are National Reference Laboratories (NRLs) or European Union Reference Laboratories (EURLs). Methods used at ANSES are generally standard methods (25%) and in-house methods (75%). These methods are developed and validated according to various national, European or international guidelines, which are generally specific to the area of interest. Furthermore, the provisions for methods of detection, quantification or confirmation are very different in different guidelines. However, standardisation and harmonisation of the overall validation process across laboratories is an important goal to achieve.

To attain this objective, a working group (WG) representing the range of expertise of laboratories was created. The skills included were: experience in qualitative (detection) and quantitative (confirmation, quantification) validation processes; experience in development and validation of methods in animal health, plant health, and food safety; quality management; and statistical approaches.

Since many ANSES laboratories are official control laboratories, they are accredited according to ISO/IEC 17025 [2015]. As a result, this standard was pivotal in establishing our internal guideline. In ISO/IEC 17025, validation is defined as “the confirmation by the examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled”. Consequently, the validation process is firstly the assessment of method performance, and secondly, the comparison with validation criteria to confirm that the methods are fit for the intended use.

At the first meeting, the WG established the roadmap to attain its objectives. It was also decided that it was necessary to have a general guideline to address the following requirements: the validation process should be harmonised, a report should be issued for the characterisation and validation of an analytical method, and guidance should be given on how to describe

FIGURE 1 / The different steps in the life cycle of analytical methods, including development, in-house validation, and reproducibility studies.



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an analytical method. This article will focus only on the general guideline.

Rapidly it became clear that there was a great deal of confusion regarding technical terms. For example, the term accuracy was confused with trueness. Linearity is also a misused term, since some analysts use the term linearity to describe the relationship between response and theoretical target (*e.g.* concentration or amount), while others use it to indicate the relationship between the calculated and theoretical target. In fact, the first is the response function and the second is true linearity.

Another term that is widely misused is sensitivity, sometimes understood as the quantification limit or the capacity of a quantitative method to detect a small variation, or the ratio of true positive results in qualitative methods.

It was therefore decided to develop a glossary. From the various guidelines, standards or official documents, 272 terms were collected, from which, after exclusion of synonyms, a glossary of 72 main terms was established. For each term, its definition was provided along with synonyms and the English translation. The main sources for the definitions were ISO Guide 99 [2007] and ISO 3534 [ISO 3534-1, 2006; ISO 3534-2, 2006].

The second step was to establish the life cycle of an analytical method [Feinberg, 2013] adapted to the objectives of French laboratories. This life cycle is presented in figure 1.

The WG clearly decided that development should not be included in the validation guideline, whereas the expression of the need should be clearly defined, as indicated by ISO/IEC 17025. As such, a chapter was specifically included in the guideline to indicate how to establish tender specifications.

The WG also decided, in compliance with official documents (*e.g.* the OIE Manual [OIE, 2014]) or other guidelines and standards, that the validation process should be performed by characteristics (trueness, precision, etc.) or by an overall approach (accuracy profile, total error) and these performances should be compared to validation criteria to determine the validity of the method. The WG defined the main characteristics to assess according to the type of analytical method (qualitative, quantitative) and the process of validation. Table 1 indicates these characteristics.

Table 1 / Main characteristics to assess depending of the type of analytical method.

Step in validation process	Characteristic		Characteristic of performance to assess depending on type of method	
			Qualitative	Quantitative
Characterisation within laboratory	Specificity [a, d]		X	X
	Sensitivity [b, e]		X	(X)
	Response function/efficacy (PCR)			X
	Precision	Repeatability	(X)	X
		Intermediate precision	X	X
	Trueness		Groundless	X
	Accuracy (trueness + precision)[c]		Groundless	X
	Linearity		Groundless	X
	Limit of	Quantification	Groundless	X
		Detection	X	(X)
	Range of validity		X	X

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Step in validation process	Characteristic	Characteristic of performance to assess depending on type of method	
		Qualitative	Quantitative
Characterisation between laboratories	Reproducibility	X	X
	Repeatability	(X)	X
	LOD	(X)	Groundless
	LOQ	Groundless	(X)
	Specificity a, d	X	Groundless
	Sensitivity b,e	X	Groundless
	Characteristics such as cost, time, ease of use, efficiency, etc. should be taken in account and indicated in tender specification	X	X

Groundless: characteristic is not relevant.

(x): characteristics in brackets are advised.

a: for qualitative methods, specificity may be analytical specificity or diagnostic specificity.

b: for qualitative methods, sensitivity may be analytical sensitivity or diagnostic sensitivity.

c: for quantitative analytical methods, accuracy is trueness and precision.

d: or false positives in some official documents.

e: or false negatives in some official documents

Certain other non-technical characteristics were also included. For example, the cost of a run, simplicity, ease of use, or duration of analysis are additional characteristics to be used to decide on method validity.

A statistical part was also developed to provide guidance on performing calculus. The main standards used were ISO 5725 [ISO 5725-1,-2, -3 and -4, 1994], ISO 3534 [ISO 3534-1 and -2, 2006] and ISO/FDIS 16140-2.2 [2016]. Annexes were also prepared to explain the statistical approaches, including basic statistics, such as how to verify normality, homogeneity of variance, estimated false positives or negatives, and why it is necessary to perform repetition to determine parameters. It was clearly explained that the performance of the method is described by characteristics (trueness, precision, accuracy) but assessed using statistical parameters (bias, standard deviation, etc.).

A specific part was included to describe how to estimate and use measurement uncertainty, based mainly on Guides JCGM 100 [2008] and ISO/TS 19036 [2006]. In addition, the possibility of estimating an uncertainty function [Gassner *et al.*, 2014] was reported.

Finally, a draft version was released to laboratories for comment and preliminary use. After a trial period of 2 months, about 200 comments were collected with 84% for the guideline. Some of these comments were about the text (45%) and others were on statistics or methodology (55%). The guideline was amended and corrected: 90% of comments were taken into account by the WG in two plenary meetings, and the final version is now currently used by laboratories.

It was also decided that it was necessary to have a “referent” for the validation of analytical methods in each laboratory and a national referent to coordinate the validation process and help analysts to use this guideline.

Finally, the process followed to establish the guideline is summarised in figure 2.

The WG worked collectively on certain general topics, such as tender specification, but also in smaller task groups for specific parts, including qualitative or quantitative methods.

In conclusion, the guideline and associated documents were established in about 20 months.

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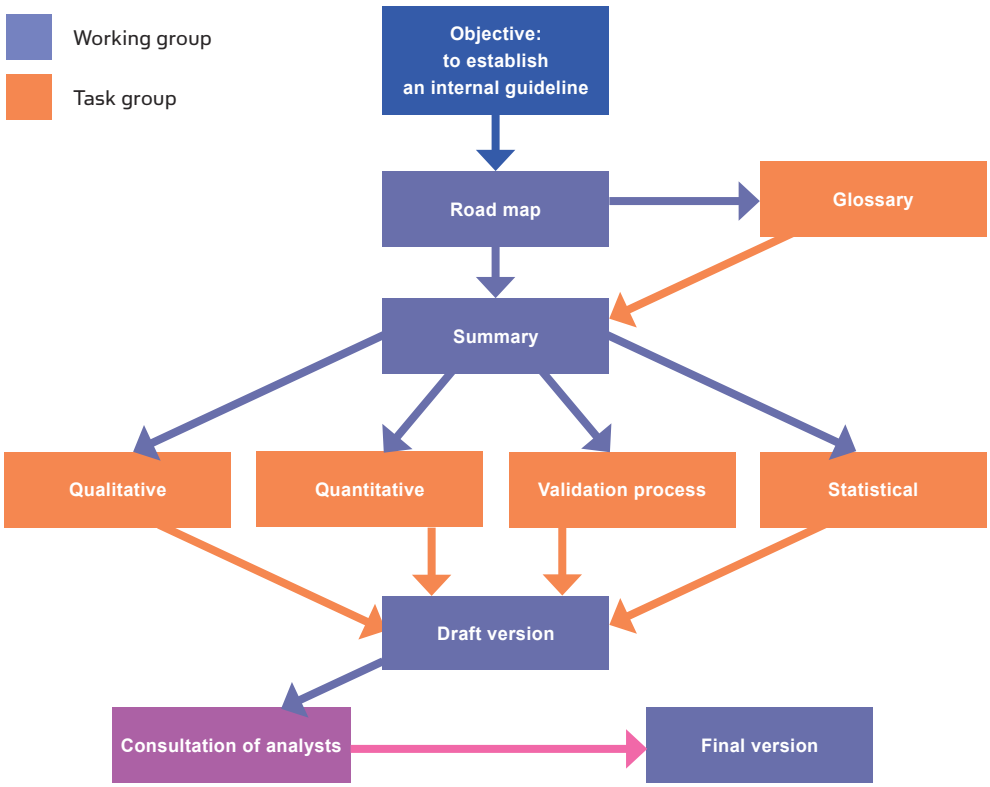
FIGURE 2 / Process to establish the guideline.



It can now be used in the various areas of interest of ANSES laboratories. This guideline does not aim to replace specific guidelines, nor official documents in animal health, plant health, or food safety, but enables harmonisation of the validation process across laboratories. The guideline also focused on the critical points in validating methods: establishing tender specifications, defining and assessing characteristics of performance, developing validation criteria, and estimating uncertainty.

(Guideline in French available at: https://www.anses.fr/fr/system/files/ANSES_GuideValidation.pdf).

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METHOD VALIDATION

Eurachem guidance on validating analytical methods

Marina Patriarca[1]*, Bertil Magnusson[2], Ulf Örnemark[3].

Abstract

Eurachem is a network of organisations within Europe that focuses on promoting the reliability of measurement results in analytical sciences and fostering good laboratory practices. To this aim, Eurachem brings together expert working groups to identify needs, pool expertise and propose authoritative guidance in the form of short information leaflets and guides. It also organises workshops to promote the exchange of experience and views among the analytical community. The validation of test methods is a key issue in ensuring the quality of analytical results, and as such is specifically addressed in standards underpinning the requirements for testing and calibration laboratories. Recently, Eurachem released a second edition of its Guide on method validation, devised to help laboratories demonstrating the fitness-for-purpose of test methods, taking into account developments in terminology, standards and analytical practice. This paper summarises this Guide and the work of Eurachem.

Keywords

- Analyses
- Eurachem
- Guidance
- Methods
- Validation

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Introduction

Eurachem (www.eurachem.org) is a network of organisations within Europe designed to (i) establish a system for the international traceability of chemical measurements and (ii) promote good quality practices in analytical sciences. Currently represented in 32 European countries, Eurachem aims to provide a forum for analytical scientists, laboratory staff and those interested in using the results of analytical measurements, to discuss common problems and develop informed and considered approaches to both technical and policy issues. Eurachem members and stakeholders meet once a year at the Eurachem General Assembly. An Executive Committee and several topical Working Groups continue pursue the organisation's stated goals throughout the year, often in cooperation with other organisations. Participation is open and channelled through the national representatives. Eurachem's main production is the development of new guidance documents, promoted through dedicated events which are also designed to provide opportunities to collect feedback. Beside the Guides, Eurachem develops information leaflets, short briefing documents on specific topics usually intended to inform a wide audience, including laboratory staff, managers and laboratory customers.

The revised Eurachem guide on method validation

Laboratory staff is well aware of the importance of validating test methods in order to demonstrate that the test results are fit for their intended use. However, what exactly should be done, why and when, is not always clear. A Eurachem Guide on method validation was first issued in 1998 and has since proven very popular. Over the years, and following the introduction of formal requirements on the competence of testing and calibration laboratories [ISO/IEC 17025, 2005], a growing body of experience has developed, leading to several changes in terminology, working practices, reference documents and requirements. Acknowledging these changes, Eurachem set up a working group on method validation to thoroughly revise the Guide [Eurachem, 2014]. This second edition accommodates the main changes in international standards and working practices, and includes notes on certain aspects of validation that are specific to qualitative test methods. While a number of field-specific guides exist, and even legal requirements on how to perform a validation study in certain areas, the Guide aims to provide a more general approach to method validation. This approach therefore supports the view expressed in ISO/IEC 17025, *i.e.* that the same requirements on competence apply to all types of testing and calibration laboratories. To this aim, the Guide includes both elements of the rationale behind validation and practical details on how to plan, perform, evaluate and make the most of such studies in the laboratories. Last, but not least, the Guide includes a substantial bibliography, listing over 80 useful sources of information. To further support method validation, the Working Group provides input for the Eurachem "Reading list", an annually updated bibliography of documents relating to several quality aspects of tests and measurements, available from the Eurachem website.

The concept of method validation

In ISO/IEC 17025, validation is defined as "confirmation, by examination and provision of objective evidence, that the particular requirements for a specific intended use have been fulfilled". From this statement, the method validation process can be designed as a sequence of steps, starting from the definition of the particular requirements for the intended use of the test result. This is the basis upon which performance requirements for specific characteristics

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of the method—such as selectivity, limit of detection and limit of quantification, working range, analytical sensitivity, trueness, precision, measurement uncertainty and ruggedness—can be set. To provide evidence that these requirements are fulfilled, the laboratory shall plan and perform a series of experiments to determine the values of these characteristics and compare them with the stated requirements. The successful outcome of such a comparison provides a statement of "fitness for purpose" for the method under scrutiny. A wealth of information is produced during a validation study. It is essential that this is properly recorded and reported to best support the fitness-for-purpose statement. Furthermore, the validation study is the best source of information on which to base the ongoing monitoring of the method's performance in routine use, thus supporting continuous adequate performance and suitable quality of the analytical results reported to customers.

Setting requirements

The purpose of method validation is to demonstrate that the test results are fit for their intended use. The first step in the process therefore entails clearly stating the analytical requirements needed to achieve this goal. The analyst has the task of translating the customer's stated or implied needs into analytical requirements. For example, if the customer's request is to determine the cadmium content in chocolate so as to state compliance with existing legal limits, it is the analyst's responsibility to assess the maximum allowable measurement uncertainty that can be associated with the measurement results and other related analytical requirements. When no other guidance exists, analysts may base their judgement on available data concerning the capabilities of the technique being used and the results of proficiency tests or other inter-laboratory studies on similar test items. Authoritative guidance on how to set requirements on target measurement uncertainty in any analytical field where this is not set by legislation or the customer is provided in another Eurachem document [Eurachem, 2015] and includes a discussion on the setting of target uncertainty for process development in addition to applied or fundamental research. Once analytical requirements have been set, the analyst can consider whether any current method can satisfy them, whether any substantial modification is needed or whether a completely new procedure has to be developed. These choices will then affect the extent of validation required.

Practical aspects of assessing method performance

The extent of validation work varies depending on the scope of the method (broad, narrow), the analytical application (qualitative, quantitative, trace or major level etc.) and measurement quality requirements such as target uncertainty. The availability of information on method performance is also a driving force in defining the extent of the experiment plan. A limited number of tests are required, for example, when introducing into a laboratory a new standard method for which performance data have already been published. In this case, the tests are designed to show that the laboratory achieves the stated precision and to check bias over the method's working range. In general, however, it is necessary to investigate several more characteristics of a test method. The limit of quantification, precision and bias (a measure of trueness) will almost always be required. The Guide provides the reader with the key definitions and rationale behind determining the various characteristics, and includes "quick reference tables" to help put this knowledge to work. Each quick reference table suggests simple experiments and provides the necessary statistical calculations for evaluating and reporting each performance characteristic. Particular attention is given to revisiting the definition and calculations to be ap-

plied to determine the limit of detection and limit of quantification. The concept and evaluation of measurement uncertainty is treated only briefly in this Guide, since Eurachem has provided comprehensive guidance on this subject elsewhere [Eurachem, 2012].

Follow-up of method validation

No matter how well the validation study is performed, important information may be lost, misused or disregarded if not recorded properly and in a way that identifies the key parameters to be kept under control when performing the test. To this aim, the Eurachem Guide includes recommendations on what and how data should be reported, along with a template for the documentation of validated methods.

Special attention is given to the use of performance data to plan internal quality controls (IQC). With reference to the Nordtest Guide on IQC [Hovind *et al.*, 2011], the method's performance can be monitored through the regular analysis of control samples, and the outcome recorded in control charts. Typically, the control value (a result obtained with a control sample) or the differences between replicates are recorded (X-charts or R- and r% charts). In both cases, the performance data on the method's precision (intermediate precision or repeatability) will form the most reliable basis for setting up the warning and action limits of the respective charts. The Eurachem Guide on method validation helps analysts make the best use of these data.

Conclusion

Nowadays, there are various guidance documents that address the validation of test methods in specific sectors. Eurachem has revised its own Guide to address changes in terminology, requirements and current practice. The Guide is intended to provide analytical laboratories with general and practical guidance on how to plan, perform and record validation studies. It also addresses the use of these data for the ongoing monitoring of method performance in routine use. The Guide is freely available from the Eurachem website.

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METHOD VALIDATION

Determination of trace and essential elements in honey by quadrupole-inductively coupled plasma-mass spectrometry

Agnieszka Nawrocka*, Maciej Durkalec, Jozef Szkoda, Miroslawa Kmiecik**.

Abstract

Honey is a nutritionally valuable food product of animal origin with a very complex chemical composition. The composition of honey depends on species of melliferous plants and the pollution of the area they were grown. It can accumulate toxic metals, which may present hazards to human health. The aim of the study was to develop a simultaneous method for determination of (aluminium (Al), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), lead (Pb), manganese (Mn), nickel (Ni), selenium (Se), silver (Ag), thallium (Tl), vanadium (V) and zinc (Zn) in honey by inductively coupled plasma mass spectrometry (ICP-MS) after microwave digestion of samples with nitric and hydrochloric acids and hydrogen peroxide solution. The results of quality controls confirmed that the described method is reliable for routine analysis of chemical elements in honey.

Keywords

- Determination
- Essential elements
- Honey
- Method development
- QICP-MS
- Trace elements

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Introduction

Honey is primarily a carbohydrate product, but it contains some organic and inorganic substances as well as trace elements. High concentrations of toxic elements in honey may result from environmental pollution and could pose a risk to the health of bees and to consumers of honey. Exposure to toxic elements could be minimized by regular control of food. Commission Regulation (EC) No 1881/2006 of 19 December 2006 established limits for lead, cadmium and mercury in foodstuffs [EU, 2006]. Based on this, the method of determining trace and essential elements in honey was developed.

This paper describes a simple method for determination of sixteen elements (aluminium (Al), arsenic (As), barium (Ba), beryllium (Be), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), lead (Pb), manganese (Mn), nickel (Ni), selenium (Se), silver (Ag), thallium (Tl), vanadium (V) and zinc (Zn)) in honey using inductively coupled plasma-mass spectrometry (ICP-MS). The analyses were performed after sample mineralization according to a microwave digestion procedure.

Equipment, reagents and certified materials

An inductively coupled plasma-mass spectrometer (ICP-MS 7700x, Agilent Technologies) equipped with a concentric nebulizer, cyclonic spray chamber, a quartz torch, and an octopole reaction system was used for all measurements. The ICP-MS was optimized daily to reach manufacturer-recommended sensitivity and stability. The standard sample introduction system was used with a glass concentric nebulizer, quartz spray chamber, and quartz torch (2.5 mm internal diameter injector). An Agilent ASX-520 autosampler system was used to deliver the samples. Standard Ni-cones were used. The operating conditions are shown in Table 1.

A Speedwave 4 (Berghof, Eningen, Germany) microwave digestion system equipped with DAK-100 high-pressure vessels made of TFM™-PTFE (pressure range: 0-100 bar) was used for sample digestion.

TABLE 1 / ICP-MS operating conditions.

Agilent Technologies 7700x			
ORS mode	No gas	He*	HEHe**
Spray chamber temperature (ºC)		2	
Nebulizer		Concentric	
RF Power (W)		1450 - 1550 W (optimized daily)	
Plasma (Ar) gas flow		15.0 L/min	
Carrier (Ar) gas flow		1.0 - 1.05 L/min (optimized daily)	
He flow rate	-	-5 mL/min	10 mL/min
Sampling depth		10 mm	
Ext 1 lens		0 V	
Ext 2 lens		-185 V	
Replicates		3	
Isotopes		^{9}Be, ^{27}Al,^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn,^{75}As, ^{80}Se,^{107,109}Ag, ^{111}Cd,^{135,137}Ba,^{205}Tl,^{208}Pb	
Internal standard		^{45}Sc,^{89}Y,^{103}Rh,^{115}In,^{159}Tb,^{165}Ho	
Correction equation		^{208}Pb=^{206}Pb+^{207}Pb	

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All solutions were prepared using Milli-Q® water (18 MΩ·cm-1) and analytical grade reagents: nitric acid HNO3 (Suprapur®, Merck, Darmstadt, Germany), and hydrogen peroxide (POCH, Poland). To prevent contamination, all glassware and plasticware were acid-washed before use. All vessels were soaked in HNO3 (10% v/v) for at least 12 hours, rinsed with distilled water, soaked for 24 hours in Milli-Q water and dried.

An ICP multi-element standard stock solution at 100 µg/mL, VAR-CAL-2 (Inorganic Ventures, USA) of Al, Ag, As, Ba, Be, Cd, Co, Cr3+, Cu, Mn, Ni, Pb, Se, Tl, Th, U, V and Zn was used to prepare the external calibration curve for each element (from 0.5 to 250 µg/L).

A multi-element stock solution (6020 ISS, 10 µg/mL) containing Bi, Li, Ho, In, Rh, Sc, Tb and Y (Inorganic Ventures, USA) was chosen as the internal standard. The tuning solution for ICP-MS 7500 cs (Agilent Technologies, USA), containing 1 µg/L of Ce, Co and Y was used to carry out daily optimisation of the mass analyser. Certified reference materials (CRMs) (SRM 1643e – trace elements in water (NIST, USA), SRM 2976 – mussel tissue, and ERM-BD150 – skimmed milk powder (IRMM, Belgium)) were used to check the accuracy of the method. High-purity argon (99.9995%) and high-purity helium (99.9995%) were supplied by Messer.

Procedure

For sample preparation, we used a procedure described by other authors [Batista *et al.*, 2012; Chudzinska and Baralkiewicz, 2011], modified as follows. Honey samples (0.3-1.0 g) were weighed into a digestion vessel. Reagent blanks were prepared by addition of Milli-Q™ water in place of the sample. For each digestion batch (8 vessels), one reagent blank was prepared to monitor the background concentration of studied elements. A volume of 5 mL of concentrated HNO_3 and 2 mL of non-stabilized 30% H_2O_2 solution was added to each digestion vessel.

Samples were left for approximately 1 h (pre-digestion) then sealed, placed in the microwave, and digested using the program described in Table 2. Afterwards they were cooled, transferred quantitatively into acid-washed polypropylene tubes, diluted to the final volume (30 mL) with Milli-Q™ water, and stored at 6±4°C in the dark until analysis. Reagent blanks were prepared under the same conditions.

TABLE 2 / Microwave digestion program.

Step	Temperature (ºC)	Pressure	Rise Time (min)	Time (min)	Power (%)
1	175	50	20	10	60
2	230	50	15	15	90
3	100	40	1	10	60
4	50	0	1	5	0

Analyses were carried out by ICP-MS in five-fold diluted samples, according to the conditions summarized in Table 1. The internal standard was delivered on line by the sample introduction system. Calibration curves were assigned using standards prepared by diluting multi-element solution. All analysed samples and standards were diluted with 5% HNO_3/1% HCl. No matrix matching of the standards to the samples was necessary.

METHOD VALIDATION

Validation

The presented method was validated according to the requirements of ISO/IEC 17025 [2005] and following guidance from the Eurachem Guide [Eurachem, 2014].

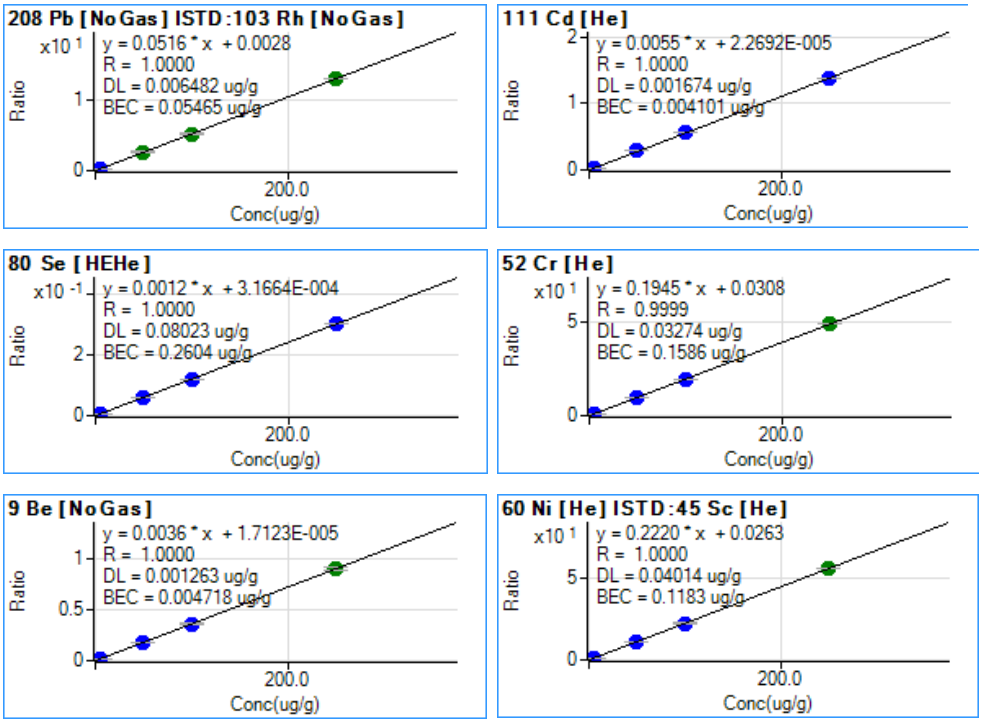
The following validation parameters were calculated: linearity, limits of detection (LOD) and quantification (LOQ), precision, and accuracy. The LOD and LOQ were calculated as a 3 or 10 times SD from results of blank matrix measurements (n=10), respectively, with a low content of the analyte. Precision was assessed using the coefficient of variation (CV with 3 independent series of 6 replicates), and accuracy was evaluated by analyte recovery. Uncertainty was estimated based on results of the validation study according to the Eurachem Guide.

Results

An effective decomposition of organic sample matrix and optimization procedures made it possible to avoid interference problems and matrix-induced ionization effects during the analysis of chemical elements in samples of food of animal origin. The LOQ was established by the determination of spiked blank samples (n=20) at the following levels: Be - 1.1, Al – 2.7, V – 1.0, Cr – 0.9, Mn – 1.1, Co – 0.8, Ni – 1.1, Cu – 1.5, Zn – 2.5, As – 0.9, Se – 1.1, Ag – 0.9, Cd – 0.8, Ba – 0.9, Tl – 1.0, and Pb – 1.3 (µg/kg).

The linearity of the calibration curves were considered satisfactory in the range 0-250 µg/L and the obtained values of determination coefficient were r2 ≥ 0.999 (Figure 1).

FIGURE 1 / Typical calibration curves obtained with the method.



METHOD VALIDATION

TABLE 3 / Process of validation determination of Cd, Pb in honey.

		Cd		Pb	
Linearity	y=ax+b	y=1,004x-0.9017		y=1.004x-0.9017	
	r	1.000		1.000	
Method LOD (µg/kg)		0.58		0.75	
Method LOQ (µg/kg)		0.79		1.29	
Concentration (µg/kg)		0.50*	19.9**	0.50*	102.0**
Repeatability	X	0.57	22.8	0.53	119.43
	s_r	0.07	1.41	0.02	8.28
	CV (%)	12.9	6.2	3.4	6.9
Within-laboratory repeatability	X	0.58	23.4	0.53	123.2
	s_r	0.04	1.56	0.02	9.12
	CV (%)	5.8	6.7	2.9	7.4
Recovery (%)	116.9	117.4	105.2	120.8	
Uncertainty	$u_c(y)$	0.07	0.03	0.02	0.79
	k	2	2	2	2
	U	0.50 ± 0.04	19.9 ± 0.05	0.50 ± 0.04	102.0 ± 1.6

*Sample fortified at 0.50 µg/kg

**19th PT on Honey, EURL-CEFAO ISS Rome

TABLE 4 / Recoveries in selected CRMs.

CRM	Value	Mn	Se	Cd	Pb	Cu	Zn	V	Co
SRM 1643e (µg/L)	Estimated	40.2	12.0	6.5	19.24	24.7	74.4	38.7	26.8
	Reference	38.97 ± 0.59	11.97 ± 0.14	6.568 ± 0.073	19.63 ± 0.21	22.76 ± 0.31	78.5 ± 2.2	37.86 ± 0.59	27.06 ± 0.31
	Recovery (%)	103.2	100.3	99.0	98.0	108.5	94.8	102.2	99.0
ERM-BD150 (mg/kg)	Estimated	0.283	0.160	0.011	0.022	1.15	37.11	-	-
	Reference	0.289 ± 0.018	0.188 ± 0.014	0.011 ± 0.0029	0.019 ± 0.004	1.08 ± 0.06	44.8 ± 2.0		
	Recovery (%)	98.0	85.3	95.0	115.7	106.2	82.8	-	-
SRM 2979 (mg/kg)	Estimated	-	1.894	0.931	1.224	4.15	142.8	-	-
	Reference	-	1.800 ± 0.15	0.82 ± 0.16	1.190 ± 0.18	4.02 ± 0.33	137 ± 13		
	Recovery (%)	-	105.2	113.5	102.9	103.1	104.2	-	-

CRM	Value	As	Ag	Cr	Ba	Tl	Be	Al	Ni
SRM 1643e (µg/L)	Estimated	59.6	1.0	20.1	572.1	7.1	13.8	156.4	61.5
	Reference	60.45 ± 0.72	1.062 ± 0.075	20.40 ± 0.24	544.2 ± 5.8	7.445 ± 0.096	13.98 ± 0.7	141.8 ± 8.6	62.4 ± 0.69
	Recovery (%)	98.6	94.2	98.5	105.1	95.4	98.7	110.3	98.6
SRM 2979 (mg/kg)	Estimated	14.46	0.012	0.434	-	-	-	144.	0.82
	Reference	13.30 ± 1.8	0.011 ± 0.005	0.500 ± 0.16				134 ± 34	0.93 ± 0.12
	Recovery (%)	108.7	91.7	86.8	-	-	-	108.2	113.4

METHOD VALIDATION

The accuracy and precision of the presented method (Table 3) were confirmed by the analysis of available CRMs and participation in proficiency tests.

Recoveries of investigated elements in CRMs are summarized in Table 4.

As a Polish National Reference Laboratory (NRL) for Heavy Metals in Food of Animal Origin and Feed, it is our duty to participate regularly in proficiency testing schemes organized by European Union Reference Laboratories (EURL-CEFAO Rome, Italy and EURL-IRMM Geel, Belgium). The results of selected proficiency tests are presented in Table 5. It is worth mentioning that our laboratory periodically organizes proficiency tests for regional veterinary laboratories dealing with the analysis of toxic elements in food of animal origin and feed.

TABLE 5 / Results of proficiency tests.

Proficiency Test	Element	Reference value (mg/kg)	Estimated value (mg/kg)	z-score σpEURL	z-score σpHorwitz
19th PT on Honey EURL-CEFAO ISS Roma	Pb	0.102	0.127	2.1	1.1
	Cd	0.0199	0.0251	2.0	1.2
IMEP-118: Determination of total As, Pb, Hg, Sn and iAs in canned food EURL-IRMM Geel	As	0.1299	0.130	0.35	0.64
	Pb	0.1043	0.104	0.63	1.27
	Cd	0.1307	0.131	0.02	0.05
22nd PT on Powdered Infant Formula EURL-CEFAO ISS Roma	Pb	0.034	0.0343	0.1	0.0
	Cd	0.0120	0.00867	-1.9	-1.3

Conclusion

This paper describes a method for the determination of trace and essential elements in food of animal origin including Cd and Pb concentrations in honey using ICP-MS. The validation parameters obtained in the validation process indicate that the proposed method presents satisfactory sensitivity, selectivity, precision, and accuracy. The results of quality controls proved that the procedure described is reliable for routine analysis of honey and other animal origin samples.

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METHOD DEVELOPMENT

Discovery and diagnosis of new viral pathogens: proposal for a generic workflow based on next-generation sequencing and new integrated data analysis approaches

Dirk Höper*, Anne Pohlmann**.

Abstract

Diagnostic metagenomic analyses gained more impact for pathogen detection and discovery in recent years due to increasing opportunities for next-generation sequencing at simultaneously decreasing prices. However, as with all novel technologies, there is a lack of standardisation in this field. But, for day-to-day routine use in a diagnostic laboratory, standardisation is urgently required. This standardisation has to take into account all steps from sampling through library preparation to data evaluation. In this short outline, some examples of metagenomics-based pathogen identification are highlighted and the most critical steps of the whole procedure are discussed. Lastly, the reader is pointed to currently ongoing initiatives that aim to achieve these goals of standardisation to pave the way for broader use of next-generation sequencing in diagnosis.

Keywords

- Data analysis
- Diagnosis
- Metagenomics
- Next-generation sequencing
- Virus

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METHOD DEVELOPMENT

Introduction

Fast and reliable detection is the basis for the discovery and control of viral infections. The established molecular methods, however, are in most cases only useful for the detection of known pathogens. During recent years, it has become more and more obvious that human and animal health is threatened by new emerging viral infectious diseases. Such diseases may spread very fast due to increasing travel and global trade chains (*e.g.* food) and transport activities. The relevance of novel diseases can easily be illustrated by several new emerging viral pathogens that were discovered over the last few years. The novel orthobunyavirus Schmallenberg virus (SBV) [Hoffmann *et al.*, 2012] or the new MERS coronavirus described in 2012 [Zaki *et al.*, 2012] have already been detected in countries around the world. New variants of influenza viruses regularly cause outbreaks globally, as for example the recent Influenza subtype H7N9 in China in 2013 [Gao *et al.*, 2013], and subtype H5N8 in Europe in 2014 and 2015 [Harder *et al.*, 2015; Verhagen *et al.*, 2015]. Influenza is a perfect example of a zoonotic infectious agent that not only causes heavy losses in poultry and swine production but also endangers human health. Examples of the detection of new zoonotic pathogens, such as the new variegated squirrel 1 bornavirus (VSBV-1) recently detected in variegated squirrels (*Sciurus variegatoides*) [Hoffmann *et al.*, 2015], underline the importance of surveillance and the search for new emerging viral pathogens in animal and human samples, as well as the need for open-view methods for fast and reliable detection and identification.

Workflow approaches

One key approach for the detection of new emerging pathogens is sequencing with next-generation sequencing (NGS) techniques. These techniques enable unbiased sequencing of all nucleic acids in a sample. Despite the fact that technical improvements and a decrease in sequencing costs over recent years have paved the way for wider use, NGS is still rarely used in day-to-day diagnosis. One major barrier is often the lack of comprehensive and easy-to-use harmonised workflows for all steps from sample to final result. Figure 1 depicts the main steps that must be included in such a stratified workflow for successful pathogen detection. This workflow must comprise methods for all steps from sampling, sample processing and sequencing, to bioinformatics tools and finely tuned methods for data analysis.

In addition, all data and the analytical results should be available in an easily comprehensible and user-friendly format. Currently, bioinformatics can still be a barrier for metagenomic pathogen detection. Bioinformatics must address use of huge data sets with respect to long-term storage and availability. More importantly, unbiased analysis which must ensure the identification of the needle in the haystack, *i.e.* finding single sequencing reads of viral origin in the overload of host sequences, is necessary. Importantly, pathogen identification does not end with the detection of sequences since this is only the starting point. For confirmation, conventional virological testing is needed, including all efforts to isolate the new virus as a prerequisite for the fulfilment of Koch's postulates. This is also the basis for further biological characterisation and development of serological diagnostics or prototype vaccines.

Over the last decade, various NGS techniques have been described [Margulies *et al.*, 2005; Bentley *et al.*, 2008; Rothberg *et al.*, 2011] and subsequently developed to commercially available products. These so-called 2nd generation NGS methods enable sequencing of nucleic acids from diverse samples even with limited DNA/RNA availability, as is for instance regularly the case with diagnostic samples. These methods require amplification of the sequencing-ready sample to ensure signal detection during sequencing. The currently available 2nd generation NGS platforms enable the sequencing of millions up to billions of individual DNA molecules simultaneously. Novel NGS of the 3rd generation [Clarke *et al.*, 2009; Eid *et al.*,

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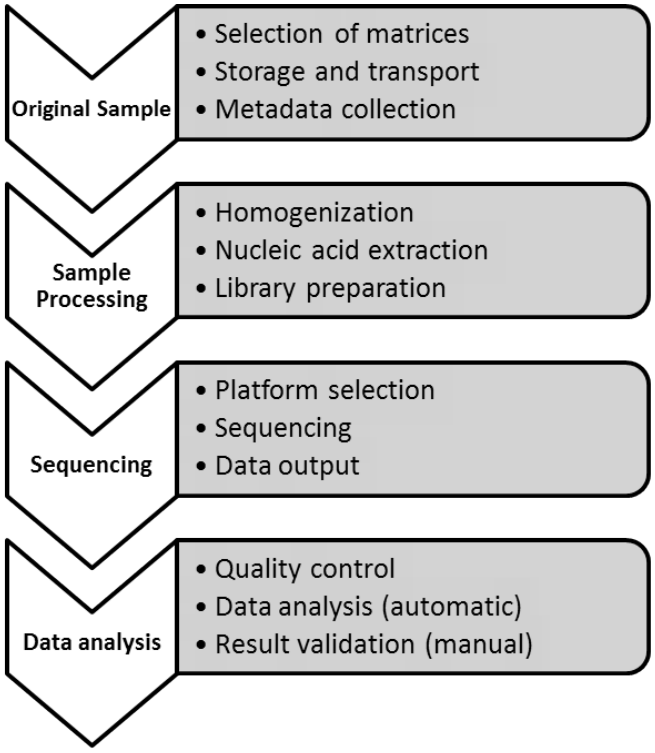
2009] requires no sample amplification for detectable signal intensity but suffers from the need for high amounts of input DNA and higher error rates, and might therefore only be instrumental for direct sequencing of samples of sufficient DNA purity and quantity.

Several examples have already proven the successful application of NGS techniques for diagnostic purposes, including discovery of new pathogens [Cox-Foster *et al.*, 2007; Palacios *et al.*, 2008; Hoffmann *et al.*, 2012; Hoffmann *et al.*, 2015], but a closer look at studies reveals that the methods used are far from being comparable, even though they are based on the same principles. This highlights the need to evaluate the different proposed procedures in order to develop a validated harmonised workflow for pathogen detection. Specifically, key steps of the workflow from sampling to sample preparation prior to NGS itself are not always carried out in an optimal way. In addition, not all steps of the procedures found in the literature are well balanced along the complete workflow, hampering the interpretation of NGS results. Key steps in this regard are sampling, including selection of sample matrices, and sample transport, storage and processing. Regarding sample selection, it is obvious that due to their different tropisms and transmission routes, pathogens are naturally not uniformly present in all sample materials like organs, serum, or swabs. Thus the selection of the sample matrix has an enormous impact on the results [Hoffmann *et al.* 2015]. It is therefore of utmost importance to include different matrices in NGS-based diagnostics. In addition, reliable and unbiased data analysis, *i.e.* screening of all sequencing reads against the complete set of available reference sequences and not only against a pre-defined subset, is crucial for any successful diagnostic implementation of NGS for pathogen detection. Most analysis strategies are essentially based on similarity comparison of the sequences gained by NGS with already available information in public databases [Bhaduri *et al.*, 2012; Naeem *et al.*, 2013; Byrd *et al.*, 2014; Scheuch *et al.*, 2015]. These similarity-based approaches limit the sensitivity of the overall process.

Moreover, the quality of the databases used is a decisive criterion for both the sensitivity and the speed of analysis. All data analysis programs/workflows have in common that they are not yet available in a harmonised and easy-to-use environment that integrates sequence data and all other available metadata, like case information with the sequence analysis results. For reliable use in day-to-day diagnosis, it is also essential that the analysis programs are continuously updated and maintained, and are regularly tested and audited.

FIGURE 1 / Principal steps of a workflow for the detection of novel pathogens based on next-generation sequencing.



Scientists and diagnosticians worldwide have recognised that the optimisation of all steps of the workflow and their integrated linkage is the prerequisite for successful implementation of virus discovery and detection by modern NGS techniques in day-to-day clinical diagnosis. Currently, globally coordinated activities dedicated to optimisation and harmonisation of the above-mentioned procedures for NGS-driven pathogen detection and in-depth characterisation are underway. Examples include the “Global Microbial Identifier” initiative (http://www.globalmicrobialidentifier.org/) and the EU-funded project “COMPARE” (http://www.compare-europe.eu/). Both tackle the present shortcomings within

powerful consortia with partly overlapping activities but with slightly different aims and visions. Together, these and other initiatives will make a substantial contribution paving the way for routine use of NGS for next-generation diagnostics.

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METHOD DEVELOPMENT

Development of a UPLC-MS/MS method for determination of mycotoxins in animal Feed

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Abstract

Mycotoxins, secondary metabolites of moulds, are natural compounds produced on vegetable raw materials, food and feed. These compounds are toxic to humans and animals. It is therefore necessary to test animal feed for contamination by mycotoxins. This testing is implemented as part of national programs for the official control of feed. The aim of this research work was to develop a multi-method for the determination of mycotoxins in feed by liquid chromatography coupled with tandem mass spectrometry.

Keywords

- Animal feed
- Determination
- Method development
- Mycotoxins
- UPLC-MS/MS

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Introduction

Mycotoxins, secondary metabolites of moulds, are natural compounds produced on vegetable raw materials, food and feed. They are toxic to humans and animals. More than 300 mycotoxins, synthesised by about 350 species of moulds, have been discovered. Initially, natural occurrence of mycotoxins was observed in foods that are most susceptible to mould growth. It has been demonstrated that mycotoxins also contaminate raw feed materials and feed [Zachariasova *et al.*, 2014]. Mycotoxin contamination of food and feed is recognised as one of the most important challenges in animal breeding and food production today [Adamse *et al.*, 2012].

It is therefore necessary to test for contamination of animal feed by mycotoxins. This testing is implemented as part of national programs for the official control of feed. Guidance values or maximum levels for mycotoxins (aflatoxins (B1, B2, G1 and G2), deoxynivalenol, fumonisins (B1 and B2), ochratoxin A, zearalenone, and T-2 and HT-2 toxin) in food and feed have been established in European Union legislation [EU, 2006].

Coexistence of even a few mycotoxins in samples and low levels in different matrixes indicate that only a multi-method based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) can provide reliable data on the contamination of feed ingredients and feed. Currently, ultra-performance liquid chromatography (UPLC) coupled with tandem mass spectrometry has the additional benefits of short analysis time, lower influence of the matrix effect, and lower detection limits.

The aim of this research work was to develop a multi-method for the determination of mycotoxins in feed using UPLC-MS/MS.

Materials and methods

■ Reagents and chemicals

Solvents: acetonitrile (analytical grade) and magnesium sulphate were obtained from POCh (Poland). Methanol (LC-MS grade) and C18 bulk sorbent were purchased from J.T. Baker (the Netherlands). Acetic acid was obtained from Sigma-Aldrich (Germany) and purified water was obtained with a Milli-Q apparatus (USA). AflaTest® immunoaffinity columns were obtained from Vicam (USA). Standards of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-AcDON), HT-2 toxin (HT-2), T-2 toxin (T-2), ochratoxin A (OTA), fumonisin B1 (FB1) and B2 (FB2), aflatoxin B1 (AFL B1), B2 (AFL B2), G1(AFL G1), and G2 (AFL G2), sterigmatocystin (STG) and zearalenone (ZEN), as well as internal standards of deoxynivalenol -13C15, zearalenone -13C18, T-2-13C24, HT-2-13C22, and ochratoxin -13C20, were purchased from Sigma-Aldrich (Germany). All standards were kept according to the recommendations of the certificates. Primary standard stock solution and working solutions were prepared in acetonitrile and the mobile phase at a concentration of 1000 µg/mL to 1 ng/mL at 2–8°C.

■ Sample preparation and extraction

Each sample was ground in a laboratory mill (Glen Mills Inc., USA), sieved (mesh size 1 × 1 mm), mixed to homogenise, and stored at ≤ -18ºC until analysis. Mycotoxins were extracted from the animal feed sample (5 g) with a mixture (20 mL) of acetonitrile:water:acetic acid (79:20:1, v/v/v) in 30 minutes (vertical shaker, 200 cycles/min). Next, the sample was centrifuged and the supernatant was divided into two aliquots. The first aliquot (used in the aflatoxins analysis) was cleaned-up with immunoaffinity columns (Aflatest, Vicam). The second, used for analysis of other mycotoxins, was cleaned-up with C18 sorbent (50 mg) and magnesium sulphate (150 mg). Extracts were evaporated, mixed with labelled internal standard solution (used for quantitation) and determined with a UPLC-MS/MS technique.

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UPLC-MS/MS analysis

A Nexera X2 system with LCMS-8050 triple quadrupole mass spectrometer (Shimadzu, Japan) and Lab Solution 1.5.2 software were used for the analysis. The mass spectrometer was operated in electrospray positive (ESI+) and negative (ESI–) ionisation mode, and two multiple reaction monitoring (MRM) transitions for each analyte were monitored. The following mass spectrometer parameters were used: resolution Q1 and Q3 - unit, nebulising gas flow – 2 L/min, heating gas flow – 10 L/min, drying gas flow – 10 L/min, interface temperature - 300°C, desolvation line temperature 250°C, heat block temperature 400°C. The chromatographic separation of mycotoxins was performed with the Luna Phenyl column (150 × 2.1 mm; particle size 3.0 µm, Phenomenex, USA) using 0.3 mL/min of constant flow and oven temperature of 40°C. The mobile phase for analysis consisted of 0.01M ammonium acetate-methanol in gradient elution. The injection volume was 10 µL.

Validation

LOD and LOQ were obtained by the analysis of background noise of 20 different blank samples (feed samples produced for different animal species and collected in different parts of Poland). The results were checked by analysis of 10 samples spiked on LOD and LOQ levels. The 20 feed samples (for different animal species and from different parts of Poland) were spiked with mycotoxins at the maximum levels (Table 2) and processed through the extraction procedure. The internal standards were used for quantitation after spiking with DON-13C15, ZEN-13C18, T-2-13C24, HT-2-13C22, and OTA-13C20. The recoveries of mycotoxins were evaluated by comparing with the concentrations found in the standard solutions. The precision of the method was measured using the same samples.

Results

During the development of the detection method, the following conditions of MS/MS were optimised for each analyte: fragmentation reactions and ionisation mode (Table 1). For most mycotoxins (except NIV, DON, 3-AcDON and ZEN), positive ionisation was applied. In the case of DON, adduct with acetic ions yielded higher intensity. For HT-2 and T-2 toxins, sodium and ammonium adducts were the options for detection, but due to lower matrix interferences ammonium adducts were preferable.

The chromatographic conditions enabled sufficient separation of mycotoxins in 18 minutes. A combination of the mobile phase and phenyl column provided acceptable peak-shapes for highly polar compounds, such as nivalenol or deoxynivalenol. The retention time for hydrophobic compounds, such as sterigmatocystin, was shorter in comparison to C18 columns (Figure 1).

Sample preparation required use of different clean-up techniques. For most mycotoxins (except aflatoxins), the extract was cleaned-up with dispersive solid phase extraction with C18 sorbent and MgSO4 salt. Due to low signal and low required maximum level (ML) for aflatoxins, immunoaffinity clean-up was necessary.

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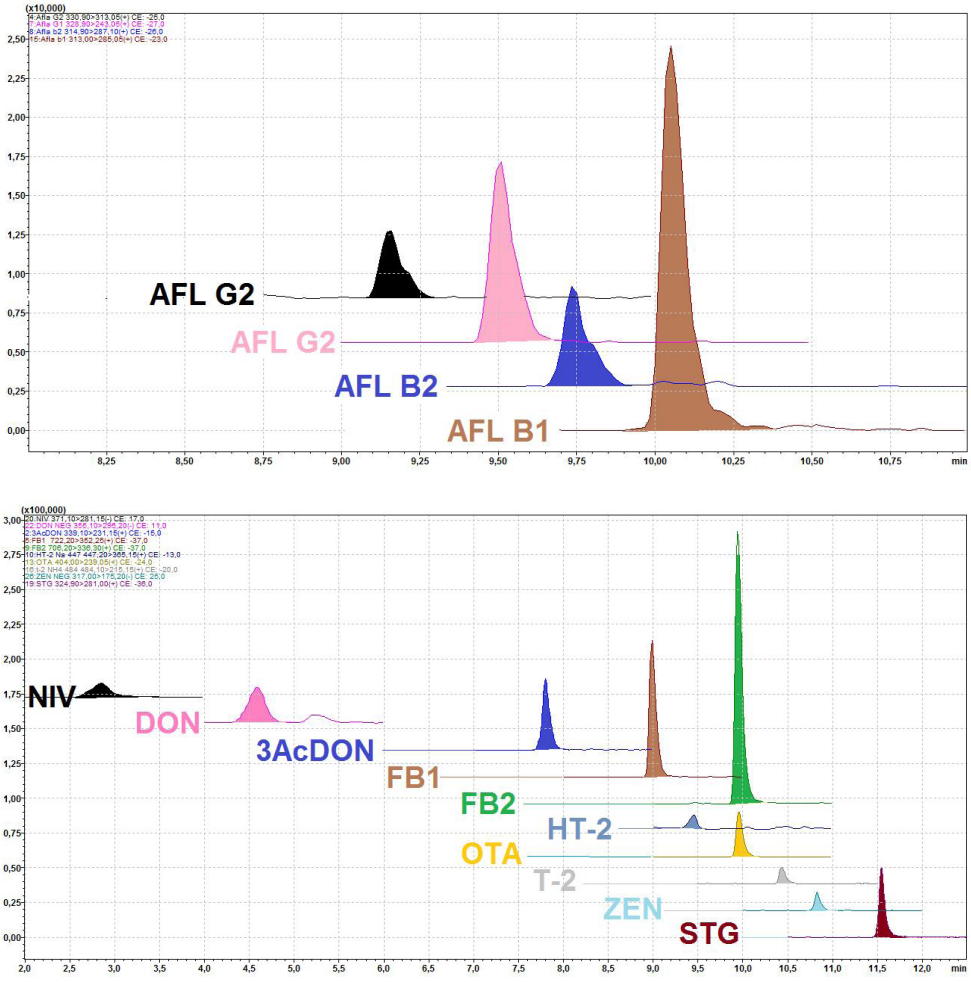
TABLE 1 / Parameters of tandem mass spectrometry detection.

Analyte	Ionisation	Parent ion	1st production	2nd production
Nivalenol	[M+CH3COO]-	371	281	223
Deoxynivalenol	[M+CH3COO]-	355	295	265
3-acetyldeoxynivalenol	[M+CH3COO]-	397	307	59
Fumonisin B1	[M+H]+	722	352	334
Fumonisin B2	[M+H]+	706	336	318
T-2	[M+NH4]+	484	305	215
Ht-2	[M+NH4]+	442	263	215
Ochratoxin A	[M+H]+	404	239	193
Zearalenone	[M-H]-	317	131	175
Sterigmatocystin	[M+H]+	325	310	281
Aflatoxin B1	[M+H]+	313	285	241
Aflatoxin B2	[M+H]+	315	287	259
Aflatoxin G1	[M+H]+	329	200	243
Aflatoxin G2	[M+H]+	331	285	245

FIGURE 1 / Ion-chromatogram of spiked feed sample (ML).



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The results of validation (Table 2) show good recovery, precision (within-laboratory reproducibility), and limit of detection, limit of quantification and usefulness of the developed procedure for multi-mycotoxin determination in animal feed.

TABLE 2 / Validation results.

	ML (·g/kg)	CVR	Recovery	LOD (·g/kg)	LOQ (·g/kg)
Nivalenol	900	18	78	50	100
Deoxynivalenol	900	15	62	50	100
3-acetyldeoxynivalenol	900	19	99	10	50
Fumonisin B1	500	17	57	10	50
Fumonisin B2	500	10	82	10	50
T-2	50	19	85	5.0	25
Ht-2	50	19	58	10	25
Ochratoxin	50	14	92	1.0	5.0
Zearalenone	100	13	96	5.0	10
Sterigmatocystin	100	18	84	5.0	20
Aflatoxin B1	5	12	95	0.50	1.0
Aflatoxin B2	5	19	85	0.50	1.0
Aflatoxin G1	5	26	75	0.50	1.0
Aflatoxin G2	5	28	86	0.50	1.0

ML – maximum level, CVR – within-laboratory reproducibility, LOD – limit of detection, LOQ – limit of quantification.

Discussion & conclusion

The issue of feed contamination by mycotoxins is well recognised. In Poland, researchers have carried out a study on the presence of 5 mycotoxins in feed materials and feed (1,255 samples over four years) [Grajewski *et al.*, 2012]. The authors found deoxynivalenol in most of the analysed samples in the concentration range 409-14470 µg/kg, and fumonisin (B1 and B2) in the range 435- 9409 µg/kg. Furthermore, they found toxins from the trichothecenes group (nivalenol, T-2 toxin and HT-2 toxin), as well as zearalenone and ochratoxin A. Authors from Belgium have developed an LC-MS/MS multi-method for the determination of mycotoxins for simultaneous determination of 23 mycotoxins in feed. Among the 82 samples of feed and feed materials, 82% were contaminated with toxins from the trichothecene and fumonisin groups. Most of the samples (75%) were contaminated with more than one mycotoxin [Monbaliu *et al.*, 2010]. Research on the occurrence of mycotoxins has also been carried out in ruminant feed (silage and hay) [Tsiplakou *et al.*, 2014]. The results showed that 13 samples tested contained aflatoxin B1 and 7 at levels higher than the limit set by European Union legislation (5 µg/kg). The problem of mycotoxins also occurs in pet nutrition (dogs and cats). In Poland, 25 dry dog foods and 24 dog foods were examined for mycotoxin contamination. In addition to commonly occurring deoxynivalenol and zearalenone, in relatively low concentrations (<120 µg/kg), the authors detected T-2 and HT-2 toxins in more than 80% of samples. The presence of these toxins in the feed is especially dangerous because of their high toxicity in cats [Błajet-Kosicka *et al.*, 2014].

As a result, a comprehensive and sensitive method for determination of mycotoxins in feed is still required. At present, only liquid chromatography coupled with tandem mass spectrometry enables control of dozens of toxic compounds in food and feed [Desmarchelier *et al.*, 2014; Jackson *et al.*, 2012; Shephard *et al.*, 2011]. The LC-MS/MS conditions used in the study

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were based on the experience of other authors [Hickert *et al.*, 2015; Malachová *et al.*, 2014].

Analysis of mycotoxins in feed samples, due to their complexity and heterogeneity, required suitable sample preparation. In our method, we used acetonitrile mixed with acetic acid solution [Dzuman *et al.*, 2014]. The real challenge was to develop a single sample preparation and effective sample clean-up method for a wide range of chemically different analytes. For the aflatoxin analysis, we had to use immunoaffinity columns, due to the low abundance of aflatoxin peaks and the low value of maximum level in feed (5.0 µg/kg). However, most authors published procedures in which this step was not necessary. For the other mycotoxins, extracts were purified with C18 sorbent, which was sufficient to reach ML levels.

The validation results show acceptable values for LOD, LOQ, recovery, and precision. Significantly lower recoveries for DON, FB1 and HT-2 (62%, 57%, and 58%, respectively) were probably caused by the matrix effect. The results of validation are highly consistent with previously published methods. The developed method is an efficient tool for the determination of mycotoxins in animal feed.

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METHOD DEVELOPMENT

Prospective study on MALDI-TOF technology for the identification and characterisation of phytopathogenic fungi

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Abstract

MALDI-TOF technology enables the identification of micro-organisms by comparative analysis of their protein spectrum to reference databases. The main objective of this study was to assess the potential of this technology for the identification of phytopathogenic fungi. Using the genus *Fusarium* as a model, we showed that MALDI-TOF analysis was a very efficient tool for identifying species, but also proved to be able to discriminate closely related *Fusarium* taxa, including species that cannot be distinguished morphologically or that only differ in their pathogenicity. However, the initial step of fungal protein extraction was shown to be of paramount importance, and the results suggest that ad hoc optimisation of this step is needed prior to the study of any fungal genus.

Keywords

- *Forma specialis*
- *Fusarium*
- Maldi-Tof
- Plant health
- Race
- Taxonomy

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Introduction

Fungal organisms (or fungi sensu lato) are uni- or multicellular eukaryotes within two kingdoms of the tree of life: Fungi (sensu stricto) and Protista. They encompass a very large variety of taxa, whose common feature is heterotrophy, and nutrition by absorption. Most of the hundred thousand species described are saprobes, playing the essential role of organic matter decomposer. On the other hand, a minority of the species act as symbiotic organisms (mycorrhizae), with mutual benefits between fungi and hosts, while more than ten thousand other species are parasites, causing diseases on plants [Agrios, 1997].

Plant diseases have a major impact both on the agricultural and forestry sectors and on the environment. It is estimated that 85% of these diseases are caused by fungi or fungal-like organisms, of which 30% are currently causing emerging diseases [Anderson *et al.*, 2004]. Control of these plant diseases requires correct and precise identification of the causal agent, in order to implement appropriate and relevant management options to suppress or at least mitigate the impact of the disease, or to adopt preventive strategies or prophylactic measures. Indeed, for a fungal genus, only a few species are true pathogens, while within a fungal species, sometimes only certain particular *formae speciales* are able to cause disease.

The identification of a fungal taxon relies on the combination of two complementary approaches: (i) observation of several morphological features of the fungus in pure culture, and (ii) analysis of the sequence of one or several regions of the genome that are considered as phylogenetic markers or 'barcodes'. Unfortunately, this dual approach does not always enable reliable assignation to a taxon name, when morphological features are not discriminant enough, or when the DNA polymorphism observed for the genetic barcode(s) is too weak between closely related taxa, such as for several species of the genus *Fusarium* [Summerell *et al.*, 2003]. MALDI-TOF (Matrix-assisted laser desorption/ionisation-time of-flight) is a technology based on mass spectrometry, analysing the protein and polypeptide composition of microorganisms to serve as a landmark for acute identification. With this technique, proteins or fractions thereof are first co-crystallised in a matrix then ionised by a laser beam (MALDI). The released ions are accelerated and separated in a vacuum tube under the action of an electric field based on their mass/charge ratio, and detected individually over time by a particle detector (TOF). The entire set of molecules will therefore be displayed as a series of peaks of different areas and heights, forming a spectrum.

The spectrum of ions is characteristic of the organism spotted on the analysis plate, and may be analysed for instance by comparison to a database of reference spectra, thus enabling identification. The proteins that are ionised and therefore used for analysis are generally ribosomal, but many other types may be released and considered for analysis (*e.g.* cell-wall proteins). MALDI-TOF technology is already widely used for the identification of pathogenic or toxigenic bacteria [Croxatto *et al.*, 2012], and serves as a routine typing tool for the ANSES Laboratory of Hydrology in Nancy. However, there have been limited reports of MALDI-TOF for the identification of fungi or fungal-like organisms to date, and most studies concern fungi of industrial or medical interest [Chalupova *et al.*, 2014]. Unlike the classical approach that combines morphological features and DNA barcode analysis and that requires several days of turnaround time, MALDI-TOF is able to generate a result within a few minutes, starting from a pure culture of the isolate to identify.

The main goal of this prospective study was to assess the potential of this promising technology for the identification of phytopathogenic fungi. First, the project focused on the optimisation of the fungal protein extraction and crystallisation steps. Second, we assessed the ability of MALDI-TOF to discriminate related taxa, from species to lower taxonomical ranks such as cryptic species, *forma specialis* or even race, in a reliable and reproducible way. Different fungal strains of the genus *Fusarium* were used as models in this study. The *Fusarium* genus comprises numerous plant pathogenic species, including cryptic species complexes

(morphologically similar and only distinguishable using a multilocus DNA sequence analysis), but also *formae speciales* or races that can only be assigned and identified based on their pathogenicity.

Materials and methods

■ Fungal cultures

All the *Fusarium* strains used in the course of this study originated from the reference culture collection of the ANSES Plant Health Laboratory. A total of 23 isolates representing 20 different species, including taxa belonging to the *Fusarium graminearum* species complex (FGSC), as well as three *F. oxysporum formae speciales* were included in the tests (Table 1). In order to assess the effect of the culture method, a panel of different *Fusarium* species was selected, and included species representing different taxonomic levels. In this respect, this panel comprised species that were highly related as well as species that were more distant from a phylogenetic point of view. The isolates of the panel were cultivated both in Petri dishes, on solid Potato Dextrose Agar (PDA, Difco), and in 5 mL of Potato Dextrose Broth (PDB, Difco) liquid medium, with constant shaking. The cultures were incubated at 22°C for 3 to 5 days, until sampling for protein extraction and MALDI-TOF analysis. Potato dextrose was the only medium tested in this study as it is widely used in mycology laboratories. For the rest of the experiments, the fungal strains were cultured according to the protocol optimised in this work, in order to provide the reference spectra after MALDI-TOF analysis.

■ Sampling of mycelium

In order to provide discriminant and reproducible MALDI-TOF spectra, the fungal protein extracts had to be as homogenous and reproducible as possible. In this respect, the effect of the technique used to collect the mixture of mycelium + asexual conidia (micro and macro-) from the fungal culture on solid agar was assessed by comparing 3 different protocols. The first strategy for sampling was adapted from Brun *et al.* [2013] and Mancini *et al.* [2013] and consisted in harvesting a 3-mm mycelial pellet by scrapping the surface of the solid culture using a sterile scalpel blade. A second technique consisted in collecting a 3-mm mycelial pellet at the centre of the culture, using a sterile needle, and carefully avoiding any scraping of agar medium (adapted from Normand *et al.* [2013]). The final method of sampling made use of a sterilised 5-mm core punch extractor. A 5-mm culture diameter disc was punched out of the fungal culture, and the agar layer underneath was removed. The mycelium disc was then cut into four equal portions, and a single one was retained for protein extraction. In addition, the effect of the age of the mycelium/conidia mixture was also investigated by sampling at different concentric circles in the culture: 0 days (active growing margin of the culture that can be sampled anytime), 3 and 5 days (based on reference circles traced every 24 h at the margin of the culture).

Each mycelium sample was individually transferred into a sterile 2-mL microtube containing 900 µL of absolute ethanol mixed with 300 µL of molecular grade water.

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TABLE 1 / List of the *Fusarium* isolates used for this study.

Species	Forma speciale	Code	Host / substrate
	-	652*#	*Triticum* sp.
F. graminearum	-	830*	*Triticum* sp.
	-	808*	*Triticum* sp.
	-	823*#	*Zea mays*
F. cortaderiae	-	824*	*Zea mays*
	-	839*	*Triticum* sp.
	-	708*	*Zea mays*
F. poae	-	861*	*Triticum* sp.
	-	878*	*Zea mays*
F. gerlachii#	-	831#	*Triticum* sp.
F. asiaticum#	-	832#	*Triticum* sp.
F. vorosii#	-	833#	*Triticum* sp.
F. acaciae-mearnsii	-	834#	*Triticum* sp.
F. aethiopicum	-	835#	*Triticum* sp.
F. mesoamericanum	-	837#	*Triticum* sp.
F. austroamericanum	-	838#	*Triticum* sp.
F. brasilicum	-	840#	*Triticum* sp.
F. meridionale	-	841#	*Triticum* sp.
F. louisianense	-	842#	*Triticum* sp.
F. ussurianum	-	843#	*Triticum* sp.
F. nepalense	-	844#	*Triticum* sp.
	-	1013	Market gardening soil
	-	1026	Banana plantation soil
F. oxysporum	*radicis cucumerinum*	1010	*Cucumis sativus*
	spinaciae	1014	*Spinacia oleracea*
	melonis	1015	*Cucumis melo*

*Isolate that was included in the test panel for protein extraction optimisation
#Cryptic species belonging to Fusarium graminearum sensu lato

■ Mycelium preprocessing

The mycelium samples were homogenised following different alternatives aimed at enhancing the release of fungal proteins, while preserving their integrity, and in order to provide reproducible data: (i) direct deposit of the mycelium with no preliminary processing [Abreu *et al.*, 2014; Marinach-Patrice *et al.*, 2009], (ii) 10 min sonication of the sample (Ultrasonic bath, Bioblock Scientific 88154), grinding for 30 s with either, (iii) 500 µL of 2-mm steel beads, (iv) two 3-mm stainless steel beads, or (v) a mixture of Grenat sand and a ceramic sphere (Lysing Matrix A, MP Biomedicals) in a multidimensional shaker set at 6.5 units (FastPrep24, MP Biomedicals) (adapted from Brun *et al.* [2013]).

■ Fungal protein extraction

After preprocessing, the microtube containing the mycelium was centrifuged for 10 min at 11 000 g. The supernatant was then carefully removed with a pipette, and the microtube was centrifuged again for 2 min at 11 000 g. The supernatant was discarded without disturbing the mycelium pellet and the microtube was kept open under a sterile flow hood until total evaporation of the liquid. The pellet was resuspended in 10 µL of 99% formic acid (Biosolve

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0614143), and the mixture was manually homogenised using a sterile pipette tip. After 5 min of incubation at room temperature, 10 µL of acetonitrile (Biosolve 01204101) were added and the sample was again homogenised with a sterile pipette tip. The mixture was mixed by 10 s vortex and centrifuged for 2 min at 11 000 g. The supernatant was then directly used as a template for MALDI-TOF and spotted on the analysis plate.

■ Spotting of fungal proteins

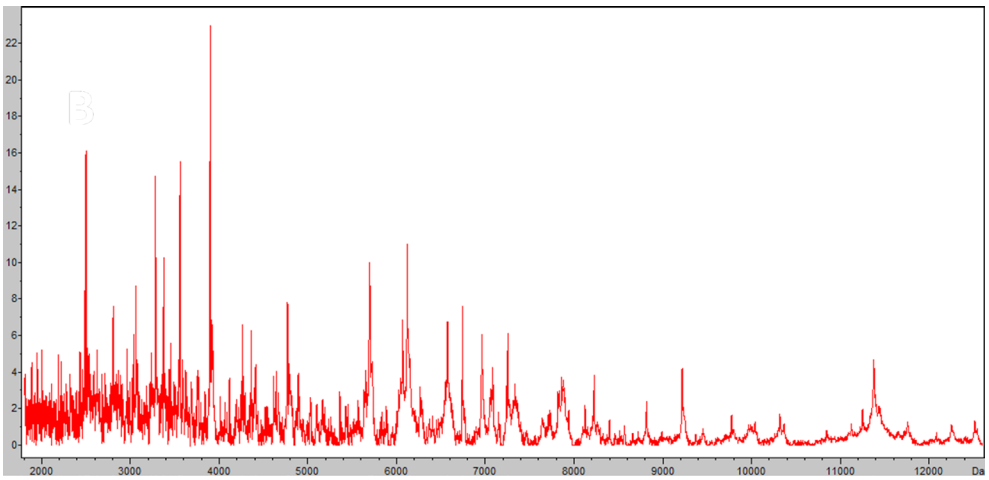
After formic acid /acetonitrile extraction, one microlitre of the supernatant was deposited onto a 96-spot MALDI-TOF plate (MSP 96 target polished steel BC, Brucker). The sample was left to dry on the bench at room temperature for 5 min, then one microlitre of matrix was added directly onto the sample, in order to activate crystallisation of the proteins. Two different types of co-crystallisation matrix were assessed and prepared according to the supplier's recommendations: alpha-cyano-4-hydroxycinnamic acid (HCCA, Brucker 8290200) and sinapinic acid (SA, Sigma-Aldrich 85429). HCCA and SA are the matrixes most frequently reported in the literature for environmental or medical fungal samples [Chalupova *et al.*, 2014].

■ Generation of MALDI-TOF reference spectra

Analyses were carried out using a Microflex MALDI-TOF spectrometer (Brucker), with default standard settings. For each fungal strain, a reference spectrum was obtained based on the mean of 24 spectra generated by 6 repetitions (*i.e.* 6 spots on the plate) for 4 independent fungal protein extractions. Each spectrum was analysed using two different software programmes. On the one hand, MALDI-Biotyper 3 (Brucker) enabled the calculation of the reference spectrum (Figure 1), and used the 70 major peaks of the spectrum for identification of the taxon by comparison with a spectrum reference database. On the other hand, two to twelve different reference spectra could be simultaneously compared, peak-by-peak by ClinProTools software (Brucker). Differences between spectra were identified based on the presence of discriminant or 'specific' peaks (present in certain strains, absent in others) and were statistically compared using principal components analysis (PCA) implemented in ClinProTools.

Results

FIGURE 1 / MALDI-TOF reference spectrum generated with *Fusarium graminearum sensu stricto* (strain LSV 652) following the preparation of fungal proteins optimised in this work.



The X-axis corresponds to the mass-to-charge ratio in Dalton, and the Y-axis to the signal intensity of the ions.

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■ Optimisation of the fungal protein extraction step

The optimal fungal protein extraction protocol was finally selected based on its ability to generate reproducible and discriminant spectra, across all the fungal strains tested. In other words, the spectra should be identical between replicate analyses and display numerous peaks, thus increasing the probability of generating discriminant peaks between different fungal taxa.

Table 2 lists the options that were finally retained for each parameter, and used for the generation of the reference spectra for all the collection fungal strains studied in this work.

TABLE 2 / Evaluation and selection of the different options during the fungal protein extraction step, prior to MALDI-TOF analysis.

Parameter	Condition
Matrix	**HCCA**
	SA
Growing condition	**Culture on solid agar**
	Liquid culture
Age of the fungal culture	**0 days**
	3 days
	5 days
Type of grinding	**None**
	Glass beads
	Stainless steel beads
	Lysing Matrix A
	Sonication
Technique for mycelium collection	**Scraping with needle**
	Scraping with scalpel blade
	Core punch extractor

The options finally retained for the preparation of reference spectra are indicated in bold.

Cultivation on solid medium was preferred based on its ability to yield more reproducible spectra and because this medium was less prone to bacterial contamination. In terms of number of peaks, no significant difference was observed between the different incubation periods before sampling. As a consequence, sampling at Day 0, or in other words, sampling mycelium samples at the growing edge of the culture, was retained, as it enabled sampling at any time during incubation of a culture. Sampling mycelium material using a sterile needle was finally chosen amongst the other harvesting procedures, despite the lower quantity of mycelium collected. Although the protocols using scalpel blade or core punch extractor enabled collection of mycelium in a standardised way, the unavoidable recovery of agar medium made them less appropriate since this caused interference with MALDI-TOF analysis and significantly reduced the number of peaks per spectrum. Amongst the different options for grinding the mycelium samples, the use of glass beads was the most efficient since it was the only way to obtain spectra with a high level of well-resolved peaks. In fact, high degradation of the fungal proteins was observed for grinding with steel beads or Lysing Matrix A, or an insufficient quantity of proteins was obtained using sonication. Nevertheless, the fungal proteins extracted without any grinding after the collection of the mycelium using a sterile needle also yielded highly reproducible spectra with numerous peaks, and this option was finally retained due to its simplicity.

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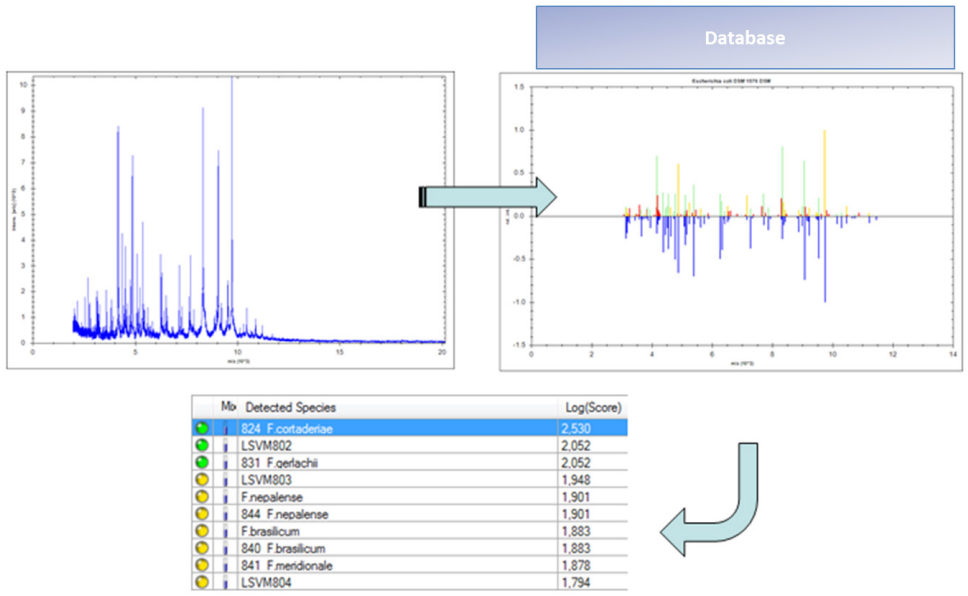
FIGURE 2 / Basic principle used for taxon identification using MALDI BIOTYPER 3 software.



The average spectrum is compared to the reference spectra database, and a homology search is conducted.

■ Identification of *Fusarium* at the species level

Overall, the majority of the ions detected in the *Fusarium* protein extracts ranged from 2 000 to 10 000 Da. Optimisation of the fungal protein extraction protocol yielded reference spectra with sufficient peak variety to discriminate the 16 different *Fusarium* species used in this study. Using MALDI-Biotyper 3 software for the comparison of the spectra to the in-house reference database produced in the framework of this study, it was always possible to assign the sample to a species, with a final log-score > 2.4 (Figure 2).

Importantly, a log-score of 1.7 is considered to be the reliability cut-off value recommended by Bruker Daltonics. In other words, the MALDI-TOF analysis of a taxon already present in the in-house database could assign a species name with a high level of certainty.

■ Identification of cryptic species of the *F. graminearum* species complex

To date, *Fusarium graminearum* sensu lato comprises 15 cryptic species, which cannot be distinguished by morphology in culture. Using the optimised protocol for sampling and extracting the fungal protein, a reference spectrum could be produced for 14 out of the 15 cryptic species. Unfortunately, in spite of repeated attempts, it was not possible to include *Fusarium boothii* in the study, since no spectrum could be generated for this species. The 14 reference spectra obtained could be analysed and compared using ClinProTools software. However, due to software limitations, it was necessary to compare them by groups of maximum seven spectra. The presence and absence of peak per spectrum for each sample were automatically assessed and the dataset was analysed by PCA. Clusters of peaks were observed on the graphic representation following principal component analyses, and were actually representative of “private” peaks observed in individual cryptic species (Figure 3). As each dot on the graph represented a peak, with associated coordinates, it was possible in return to select the discriminant peaks directly on the spectrum using ClinProTools.

Direct comparison of the different spectra confirmed that the private peaks were indeed present for some of the cryptic species, whereas they were absent in others, thus enabling discri-

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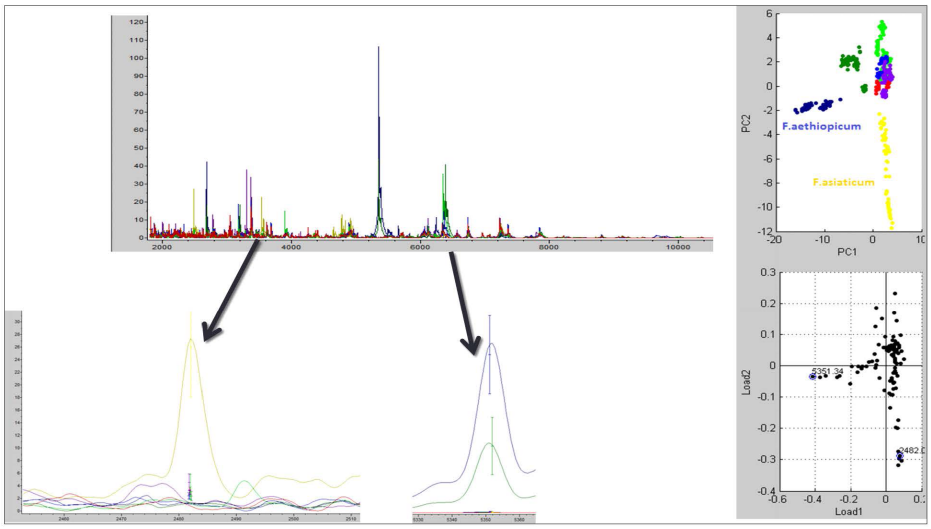
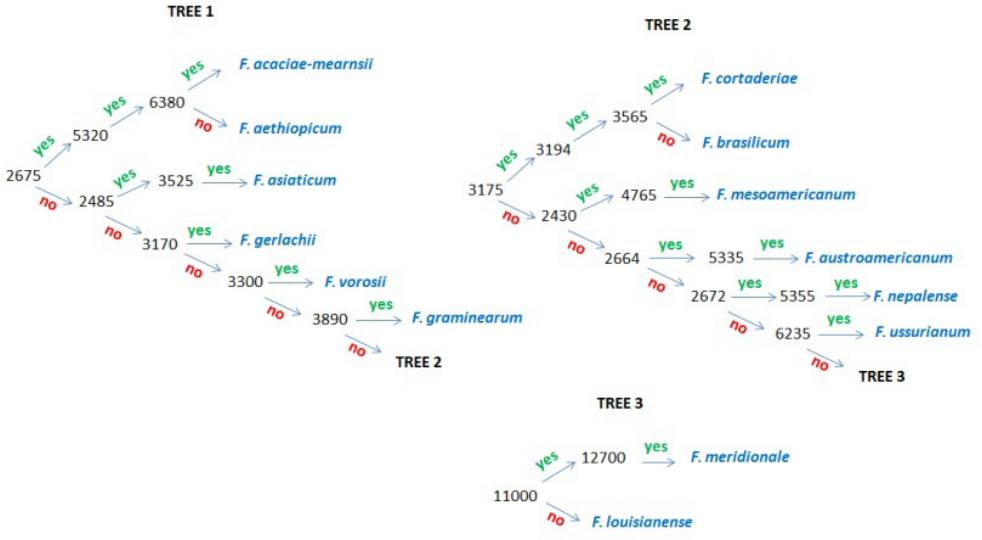
FIGURE 3 / Comparative analysis of MALDI-TOF spectra generated with several cryptic species of the *Fusarium graminearum* species complex, and associated principal components analysis enabling identification of potentially species-specific peaks.



mination of cryptic species. By combining the presence/absence of the different discriminant peaks that were identified, it was possible to define a pattern for each of the 14 cryptic species analysed, following a flow diagram, and to distinguish all species based on their spectrum (Figure 4).

■ Identification of *Fusarium oxysporum formae speciales*

FIGURE 4 / Flow diagram for the identification of *Fusarium graminearum sensu lato* cryptic species, based on the unique combinations of presence/absence of discriminant peaks in the MALDI-TOF spectra. The unique peaks were picked out by parallel analysis of the spectra using ClinProTools.



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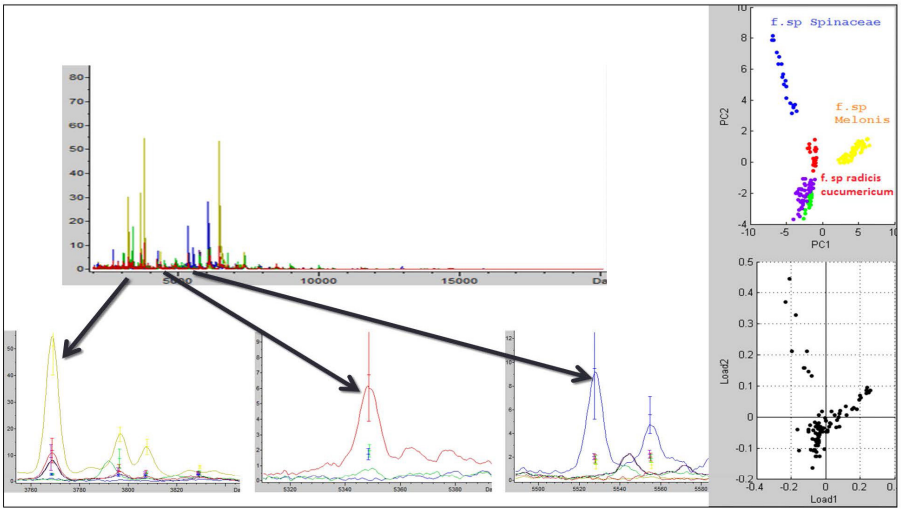
In the framework of this preliminary study, we limited the panel of *Fusarium oxysporum formae speciales* to three taxa (f.sp. *melonis*, f.sp. *radicis cucumerinum*, and f.sp *spinaciae*, pathogenic on melon, cucumber and spinach, respectively) and following the same approach described for cryptic species discrimination, it was possible to differentiate the *formae speciales*. We identified peak combinations whose presence or absence enabled us to distinguish the taxa (Figure 5).

FIGURE 5. Comparative analysis of MALDI-TOF spectra generated with three *Fusarium oxysporum formae speciales*, and associated principal components analysis enabling identification of discriminant peaks for f. *sp. melonis*, *radicis-cucumerinum* and *spinaciae*, respectively.



Discussion

This prospective study showed that after a preliminary optimisation step, MALDI-TOF was a powerful technique to identify and distinguish *Fusarium* species, and also *Fusarium* taxa at lower taxonomic levels such as cryptic species and *formae speciales*. MALDI-TOF mass spectrometry is a technology that is more and more widely used in bacteriology, and has proved to be particularly useful and accurate for identification and typing of strains [Bizzini and Greub, 2010]. The reliability and the robustness of the results depend entirely on the exhaustiveness of the database of reference spectra which is used by the associated analysis software. Several databases are available and can be purchased directly from the manufacturer. However, several suppliers of MALDI-TOF equipment are present on the market, and unfortunately, the databases are not transferable from one brand to another. Another issue is that the databases currently available are somewhat specific to medical or environmental applications. To date, Bruker Daltonics proposes two different sets of data to be used with the Micro Flex equipment, one for bacterial identification (Bruker Taxonomy/Bacteria) and another for eukaryotic organisms, including several genera of fungi (Bruker Taxonomy/Eukaryota). Unfortunately, the second one only contains a very limited set of phytopathogenic fungi, which can be easily explained by the fact that up to now the use of MALDI-TOF technology for plant pathology was scarcely reported in the scientific literature. This meant that in our case, the reference database had to be produced in-house. Only a few protocols are described in the

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literature regarding fungal protein extraction for MALDI-TOF analysis, and even fewer report efforts for standardisation.

The preliminary results of this prospective study focused on plant pathogenic fungi first confirmed that the availability of a comprehensive dataset was mandatory for reliable identification. In order to discriminate the *Fusarium* species that were used as a model in this study, it was necessary to implement an in-house database containing reference spectra. As a direct consequence, it was first mandatory to work on optimisation and standardisation of the fungal protein extraction step, and to produce robust reference spectra with repeated measures. Preliminary attempts to use the protocol suggested by the manufacturer, *i.e.* using liquid culture cultivation and a simple ethanol extraction method, were unsuccessful in producing good quality and repeatable spectra with our set of fungi. The disruption of mycelium and the rough extraction of proteins appeared to be more difficult than with prokaryotes, probably because the cell walls are in general far more resistant. We also observed that numerous parameters might greatly influence the rate of recovery and the quality of the fungal proteins before ionisation. Some of the options that were tested in our context, such as the use of SA as a matrix or the use of an unsuitable grinding technique, did not enable us to recover any ionisable proteins at all for some strains tested, which may be explained by either a poor level of protein release in the solution, their destruction or fragmentation, or their recovery with inappropriate polarity.

Moreover, given the extraordinary richness of the taxa within the Fungi kingdom, it was necessary to set up a protocol that combined high reproducibility and the capacity to discriminate by generating spectra containing a massive quantity of information, to improve the odds of identifying discriminating peak combinations.

The protocol that was optimised here for the efficient ionisation of fungal proteins in the genus *Fusarium* was also evaluated with several other genera of fungi (Monilinia spp., Colletotrichum spp.) or oomycetes (Phytophthora spp.), which include numerous aggressive plant pathogens. Unfortunately, the spectra that were obtained were poorly reproducible (data not shown), which suggested that the recovery rate for fungal proteins varied considerably across genera, probably depending on the chemical composition of the fungal cell walls. Our results confirmed the hypothesis that for each type of microorganism, and even for each genus of fungi, preliminary work had to be done to optimise the steps leading to the extraction and purification of the set of proteins before ionisation and MALDI-TOF analysis [Chalupova *et al.*, 2014]. As a result, it is unfortunately unlikely that a common and standardised protocol could be developed and used across the fungal kingdom.

However, provided that an appropriate extraction protocol is available, our preliminary results with *Fusarium* showed that MALDI-TOF analysis was quite promising for the identification of taxa, even at very low taxonomical levels.

The initial results of Marinach-Patrice *et al.* [2009] have already demonstrated that MALDI-TOF analysis enabled the identification of several *Fusarium* species involved in human dermatitis, but these were genetically distant. The results obtained here highlight the potential of MALDI-TOF to discriminate even closely related taxa, such as cryptic species for the genus *Fusarium* and are in line with the recent conclusions of Al-Hatmi *et al.* [2016] regarding the *F. fujikuroi* species complex. These cryptic species can only be identified and distinguished based on sequence analysis of several DNA markers, which requires several days of turnaround time. Likewise, through statistical comparison of the spectra, specific combinations of presence/absence of exclusive peaks made it possible to distinguish among several *formae speciales* within *F. oxysporum*. *Forma specialis* and race represent two particular taxonomic levels only observed in certain fungal species such as *F. oxysporum*, which are directly related to the pathogenicity towards specific host plants, or a particular set of varieties of the same host plant, respectively. The identification of the *forma specialis* or race typically requires several days or weeks of pathogenicity testing on plants, and MALDI-TOF analysis would

provide a quicker and easier means of obtaining a result. Following our work, preliminary attempts to discriminate races within a *forma specialis* yielded very promising results with *F. oxysporum* f. sp. *cubense* (Foc), causing the destructive Panama disease on banana trees (data not shown). Several races of Foc are described based on their pathogenicity towards certain banana varieties. One of them, *i.e.* Tropical Race 4 is currently threatening the Cavendish variety, which is the most cultivated banana cultivar throughout the world, and therefore accurate identification of this race is of paramount importance for the enforcement of disease management strategies. However, several strains of the same race, with different geographical origins, should be collected and assessed in order to confirm the potential of MALDI-TOF analysis.

Testing a larger set of *Fusarium formae speciales* and races would of course be necessary for a comprehensive assessment of the reliability of MALDI-TOF technology. However, preliminary results are definitely promising and we may be optimistic about the usefulness of MALDI-TOF for acute identification, at very low taxonomic levels. It may also be imagined that on some occasions and after a thorough comparison, identification of *formae speciales* and races would no longer require pathogenicity testing, thus saving time and money. Of course, by using software that enables a comparison of spectra using a peak–by-peak approach, such as ClinProTools, peaks representing proteins or portions thereof might be connected to pathogenicity features.

As a conclusion, this preliminary study supports the potential of MALDI-TOF for the identification and the acute characterisation of phytopathogenic fungi, taking the *Fusarium* genus as a model. The results also suggest that prior optimisation of the protein extraction step is a prerequisite for MALDI-TOF analysis of different fungal genera, which will make it difficult to build a universal fungal reference database. Once the initial investment has been made for the purchase of the equipment, routine analyses using MALDI-TOF are very affordable in terms of reagents and consumables, and enable simultaneous processing of more than 90 samples within less than 2 hours, which is undoubtedly an advantage over the currently available identification techniques.

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DISPATCHES

Summary of the 3rd Workshop for Heads of Plant Pest Diagnostic Laboratories.

Françoise Petter*, Madeleine McMullen**.

In the framework of its diagnostic activities, the European and Mediterranean Plant Protection Organisation (EPPO) regularly organises conferences and workshops. Since 2013, three workshops have been organised to provide a forum for heads of laboratories to meet and exchange experiences on horizontal problems faced in their laboratories. The Third EPPO Workshop for Heads of Plant Pest Diagnostic Laboratories was organised at the Plant Pathology Research Centre, Rome (08-11 September 2015). The meeting was attended by 26 heads or deputy heads of laboratories from 19 countries. A short summary of the main topics discussed during this workshop is presented.

Accreditation with a flexible scope for plant pest diagnostic laboratories

Historically, the accreditation of laboratories has usually been based on a fixed scope which should define clearly and unambiguously the range of tests covered by the laboratory's accreditation (*e.g.* immunofluorescence test for the detection of *Ralstonia solanacearum* on potato tubers). However, this does not readily allow new or modified tests to be added to a laboratory's scope, even when the competence of the laboratory in performing and validating related tests has already been evaluated by an accreditation body. Although applications for an extension to scope can be made at any time, the timescales involved may actually prevent quick reactions to client's demands. Consequently, the concept of flexible scope has been developed [EPPO, 2014].

A flexible scope of accreditation allows a laboratory to undertake certain tests, and to report the results as accredited, even though these tests are not explicitly stated in the laboratory's scope (see Requirements for the Accreditation of Flexible Scopes [EA, 2008]. Examples of situations where the need for flexible scope may arise are:

- Optimisation of a given test
- Modification of an existing test to broaden its applicability (*e.g.* to deal with new matrices)
- Inclusion of a test equivalent to the one that is already covered by accreditation.

The concept of flexible scope encompasses a degree of flexibility which is usually agreed in consultation with the accreditation body [EPPO, 2014]. Presentations from different laboratories on their approach to flexible scope showed that, when granted, flexible scope can be established at different levels (*e.g.* flexible scope with a recognised method; possibility within

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the flexible scope to adopt, adapt and/or develop new tests; and flexibility on all diagnostic activities). Given this heterogeneity of approaches, the heads of laboratories considered that regional harmonisation is essential and that discussions should continue between EPPO and the European Co-operation for Accreditation (EA). It was suggested that a joint meeting with representatives of plant health laboratories and accreditation bodies would be needed to prepare a document on a joint approach.

Q-bank

Q-bank is a database on quarantine pests which originally started as the result of a Dutch project to strengthen plant health infrastructure. Discussions are in progress to consider whether this database should be hosted within EPPO in the future, and in order to facilitate these discussions, a questionnaire on its use and the needs of laboratories was sent to all laboratories involved in official diagnostics. The results of the survey were presented during the workshop.

The workshop participants agreed with the results of the survey that the focus for integration into the EPPO system should be:

- DNA sequences for blasting[1]
- Protocols for barcoding, and
- Where to find biological material.

The workshop emphasised the importance of these data (and of their maintenance) as they are key to the work of laboratories, in particular reference laboratories in the EPPO region. Other types of information currently present in Q-bank, such as geographical distribution, host plants, keys, pictures etc., are readily available in EPPO datasets (*e.g.* the EPPO global database https://gd.eppo.int/, EPPO diagnostic protocols).

The workshop supported the idea that a project for integration of Q-bank data should be proposed to the EPPO Council in order to ensure long-term sustainability of access to the data.

However, it was also noted that progress on the integration would still be dependent on identifying sources of funding for the project.

A review of the outcomes of the Q-collect project on collections of biological material for plant health diagnostics

Q-collect is an EU FP7-funded project (1 October 2013-30 September 2015) with the aim of improving the status of (reference) collections important to plant health (more information on the project is available on the Q-collect website, www.q-collect.eu).

The objectives of the project were to:

- make an inventory of existing plant pest collections in the European and Mediterranean region, and of their content;
- develop guidelines for quality standards, (later to be proposed as EPPO Standards);

1 The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The programme compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

- develop guidelines to improve the accessibility of these collections;
- design and build a network of reference collections;
- develop an info-portal on the internet to provide information on the outcomes of the project;
- disseminate results to stakeholders;
- support a network of national reference collections relevant to national and EU plant health policy;
- provide guidelines for preserving, maintaining and improving the quality and accessibility of national reference collections (specimens, tissues and DNA) and consequently ensuring harmonisation of collection maintenance across Europe.

The main findings of the Q-collect project were as follows:

- Most collections are research or working collections and only a few of them are organised to provide services to outside users;
- There is a limited amount of information on collection holdings available online;
- Many collections have no quality system in place and accreditation of collections is rare;
- Sharing of material between collections to ensure resilience is not common and can be considered as a high risk for loss of important biological material in case of incidents with buildings or equipment;
- Appropriate basic funding is not secure enough and there is a need for a common policy on collection management throughout the region.

Proposals made during the Q-collect project to improve the current situation were presented:

- Guidelines on quality assurance for reference collections: it was noted that these should be further developed in the framework of the EPPO specialised Panels on diagnostics for each group of organisms;
- Criteria for the establishment of a sustainable network: it is proposed to establish a network to bring together previously dispersed information on biological material and promote collaboration. Criteria for reference and working collections were proposed;
- Plans for an online platform to foster and facilitate networking and data sharing were presented. The objective of setting up this type of platform is to improve accessibility and visibility of biological material (and related information) available in collections.

Heads of laboratories discussed the question of sharing of material from collections. Participants with various working collections, for example from Denmark, Hungary, Latvia, Russia, and Switzerland, considered that sharing the material in their possession was important. They were also convinced that, as a first step, having the information about their collections displayed through a web-portal is essential. The workshop concluded that a pilot platform including a deposit form for biological material should be developed. The workshop also recommended that the criteria proposed for the establishment of a sustainable network should also be reviewed in the EPPO Panels on diagnostics. It was also suggested that the guidelines developed within the Q-collect project should be sent for information to the organisers of test performance studies, as these are laboratories sharing biological material from their collections in this framework. This will allow feedback to be gathered about the criteria proposed for the network.

An analysis of the impact of the 'Nagoya Protocol' for collections was presented. The workshop noted that this legislation seems to create conflict with obligations under the International Plant Protection Convention (IPPC), for example it is not clear whether specimens isolated from imported consignments can be stored and used for diagnostic and research purposes.

Finally, the heads of laboratories reviewed and endorsed a white paper summarising the outcome of project proposals and highlighting the main challenges ahead. This paper is available at: http://archives.eppo.int/MEETINGS/2015_conferences/q-collect/White_paper_on_collections_2015-10.pdf

All presentations are available on the EPPO Website: http://archives.eppo.int/MEETINGS/EPPO_workshops.htm

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