VALIDATION & VERIFICATION OF CHEMICAL TESTING METHODS

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Introduction

Aquatic environment receives influx of pollutants from many point and non-point sources and considered ultimate sink in the pollutant transport cycle. Other than the environmental pollutants, pesticides and antibiotics are often directly used in aquaculture. Hence, a diverse range of anthropogenic chemicals such as Agricultural pesticides, Antibiotics, Vet drugs, Poly aromatic hydrocarbons, PCBs, Dioxins, Natural Toxins, Formaldehyde, PPCPs, PFAS, PBDE etc. are tested in various fish and fisheries products to ensure food safety and safeguard public health. A typical test method of chemical contaminants analysis involves extraction, cleanup, pre-concentration/dilution, and finally instrumental analysis. All sample preparation and processing procedures should be undertaken within the shortest time practicable to minimise sample decay and pesticide losses. Analyses for residues of very labile or volatile pesticides should be started, and the procedures which could lead to loss of analyte should be completed as soon as possible, but preferably on the day of sample receipt. Sample preparation, sample processing and sub-sampling to obtain portions should take place before any visible deterioration occurs. Only the edible part of the produce is sampled and tested. A clean-up, or dilution step may be necessary to reduce matrix interferences and reduce contamination of the instrument system leading to an improved selectivity and robustness.

Sample extracts are normally analysed using capillary gas chromatography (GC) and/or high performance or ultra-performance liquid chromatography (HPLC or UPLC) coupled to mass spectrometry (MS) for the identification and quantification of pesticides in food and feed samples. Various MS detection systems can be used, such as a single or triple quadrupole, ion trap, time of flight or orbitrap. Typical ionisation techniques are: electron ionisation (EI), chemical ionisation (CI), atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). Different acquisition modes may be used such as full-scan, selected ion monitoring (SIM), selected reaction monitoring (SRM) and multiple reaction monitoring (MRM). Nowadays, selective detectors for GC (ECD, FPD, PFPD, NPD) and LC (DAD, fluorescence) are less widely used as they offer only limited specificity.

Since the validity of the test result is critical for regulatory control and protecting public health, it is important the test methods are rugged, repeatable, and reproducible. Laboratories often use official test methods of AOAC, AOCS, APHA, USEPA, FDA etc. or even use in house developed method. In house methods needs to be completely validated and official methods must be verified for the intended use in the laboratory. Hence, harmonized protocols for method validation and verification is crucial to ensure reliability of test results produced in different laboratories, in different parts of the world. The Document Nº SANTE/12682/2019, Commission Implementing Regulation (EU) 2021/808, and the Eurachem guideline for method validation are considered gold standards for method validation and verification of analytical food testing methods.

What is Method Validation?

Method validation is the process of confirming the method has performance capability as the application requires. It is a process of demonstrating or confirming that a method is suitable for its intended purpose which can be qualitative analysis, quantitative analysis, screening analysis, confirmatory analysis, limit tests, matrix extensions, platform extensions, and emergency/contingency operations. Validation includes demonstrating performance characteristics such as accuracy, precision, sensitivity, selectivity, limit of detection, limit of quantitation, linearity, range, and ruggedness, to ensure that results are meaningful and appropriate to make a decision. Following table lists the various definitions provided by different regulatory agencies.

References	Validation Definition
Codex CAC/GL 74	Process to establish the **performance characteristics** and limitations of an analytical method: which analytes, in what kind of matrices, in the presence of which interference. Result = precision and trueness values of a certain analytical method under the examined conditions.
ISO 16140-1	Establishment of the **performance characteristics** of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled.
USDA FSIS	Process to measure **performance characteristics** of a particular test, with the goal of determining whether the test is equivalent to the reference test for the intended conditions of use. "Equivalent" = the performance characteristics are statistically indistinguishable.

US FDA	Demonstration that adequate confidence is provided when the results obtained by the alternative method i.e. the commercially available kit, are comparable to or exceed those obtained using the reference method using the **statistical criteria** contained in the approved validation protocol.
Health Canada	Evaluation of the **performance parameters** of a new method in comparison to an accepted reference method using paired or unpaired samples. In the context of relative validation, the results of the reference method are assumed to reflect the true microbiological status of the samples and the performance parameters of the alternative method are calculated in relation to this.
ISO 17025:2005	The confirmation by examination and the provision of objective evidence that the **particular requirements** for a specific intended use are fulfilled.

Identification of analytes and confirmation of results

Identification

Mass spectrometry coupled to chromatography

Mass spectrometry coupled to a chromatographic separation system is a very powerful combination for identification of an analyte in the sample extract. It simultaneously provides retention time, mass/charge ratios and relative abundance (intensity) data.

Requirements for chromatography

The minimum acceptable retention time for the analyte(s) under examination should be at least twice the retention time corresponding to the void volume of the column. The retention time of the analyte in the extract should correspond to that of the calibration standard (may need to be matrix-matched) with a tolerance of ±0.1min, for both gas chromatography and liquid chromatography. Larger retention time deviations are acceptable where both retention time and peak shape of the analyte match with those of a suitable IL-IS, or evidence from validation studies is available. IL-IS can be particularly useful where the chromatographic procedure exhibits matrix induced retention time shifts or peak shape distortions. Overspiking with the analyte suspected to be present in the sample will also help to increase confidence in the identification.

Requirements for mass spectrometry (MS)

MS detection can provide mass spectra, isotope patterns, and/or signals for selected ions. Although mass spectra can be highly specific for an analyte, match values differ depending on the particular software used which makes it impossible to set generic guidance on match values for identification. This means that laboratories that use spectral matching for identification need to set their own criteria and demonstrate these are fit-for-purpose. Guidance for identification based on MS spectra is limited to some recommendations whereas for identification based on selected ions more detailed criteria are provided.

Recommendations regarding identification using MS spectra

Reference spectra for the analyte should be generated using the same instruments and conditions used for analysis of the samples. If major differences are evident between a published spectrum and the spectrum generated within the laboratory, the latter must be shown to be valid. To avoid distortion of ion ratios the concentration of the analyte ions must not overload the detector. The reference spectrum in the instrument software can originate from a previous injection (without matrix present), but is preferably obtained from the same analytical batch.

In case of full scan measurement, careful subtraction of background spectra, either manual or automatic, by deconvolution or other algorithms, may be required to ensure that the resultant spectrum from the chromatographic peak is representative. Whenever background correction is used, this must be applied uniformly throughout the batch and should be clearly recorded.

Requirements for identification using selected ions

Identification relies on the correct selection of ions. They must be sufficiently selective for the analyte in the matrix being analysed and in the relevant concentration range. Molecular ions, (de)protonated molecules or adduct ions are highly characteristic for the analyte and should be included in the measurement and identification procedure whenever possible. In general, and especially in single-stage MS, high m/z ions are more selective than low m/z ions (e.g. $m/z < 100$). However, high mass m/z ions arising from loss of water or loss of common moieties may be of little use. Although characteristic isotopic ions, especially Cl or Br clusters, may be particularly useful, the selected ions should not exclusively originate from the same part of the analyte molecule. The choice of ions for identification may change depending on background

interferences. In high resolution MS, the selectivity of an ion of the analyte is determined by the narrowness of the mass extraction window (MEW) that is used to obtain the extracted ion chromatogram. The narrower the MEW, the higher the selectivity. However, the minimum MEW that can be used relates to mass resolution. Extracted ion chromatograms of sample extracts should have peaks of similar retention time, peak shape and response ratio to those obtained from calibration standards analysed at comparable concentrations in the same batch. Chromatographic peaks from different selective ions for the analyte must fully overlap. Where an ion chromatogram shows evidence of significant chromatographic interference, it must not be relied upon for identification. Different types and modes of mass spectrometric detectors provide different degrees of selectivity , which relates to the confidence in identification. The requirements for identification are summarised in the following Table.

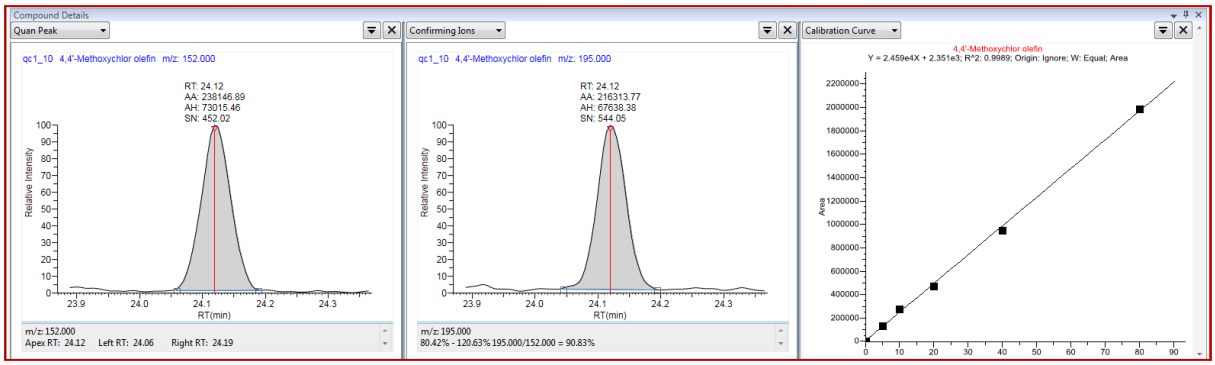
MS detector/Characteristics		Acquisition	Requirements for identification	
Resolution	Typical systems (examples)		Minimum number of ions	other
Unit mass resolution	Single MS quadrupole, ion trap, TOF	full scan, limited m/z range, SIM	3 ions	S/N ≥ 3 Analyte peaks from both product ions in the extracted ion chromatograms must fully overlap. Ion ratio from sample extracts should be within ±30 % (relative) of average of calibration standards from
	MS/MS triple quadrupole, ion trap, Q-trap, Q-TOF, Q-Orbitrap	selected or multiple reaction monitoring (SRM, MRM), mass resolution for precursor-ion isolation equal to or better than unit mass resolution	2 product ions	

				same sequence
Accurate mass measurement	High resolution MS: (Q-)TOF (Q-)Orbitrap FT-ICR-MS sector MS	full scan, limited m/z range, SIM, fragmentation with or without precursor-ion selection, or combinations thereof	2 ions with mass accuracy ≤ 5 ppm	S/N ≥ 3d Analyte peaks from precursor and/or product ion(s) in the extracted ion chromatograms must fully overlap

Method validation parameters and acceptance criteria

Sensitivity/Linearity

Sensitivity of a method implies the lowest possible concentration the method can quantify with satisfactory repeatability and reproducibility. For quantitative analysis a calibration curve is prepared by injecting matrix matched or procedural standards spiked at different concentrations. The following Figure illustrates a typical example:



The lowest calibration level (LCL) must be equal to, or lower than, the calibration level corresponding to the RL. The RL must not be lower than the LOQ. Bracketing calibration must be used unless the determination system has been shown to be free from significant drift, e.g. by monitoring the response of an internal standard. The calibration standards should be injected at least at the start and end of a sample sequence. If the drift between two bracketing injections of the same calibration standard exceeds 30 % (taking the higher response as 100 %) the bracketed samples containing pesticide residues should be re-analysed. Results for those samples that do not contain any of those analytes showing unacceptable drift can be accepted

provided that the response at the calibration level corresponding to the RL remained measurable throughout the batch, to minimise the possibility of false negatives. If required, priming of the GC or LC system should be performed immediately prior to the first series of calibration standard solutions in a batch of analyses. The detector response from the analytes in the sample extract should lie within the range of responses from the calibration standard solutions injected. Where necessary, extracts containing high-level residues above the calibrated range must be diluted and re-injected. If the calibration standard solutions are matrix-matched, the matrix concentration in the calibration standard should also be diluted proportionately. Multi-level calibration (three or more concentrations) is preferred. An appropriate calibration function must be used (e.g. linear, quadratic, with or without weighing). The deviation of the back-calculated concentrations of the calibration standards from the true concentrations, using the calibration curve in the relevant region should not be more than ±20 %. Calibration by interpolation between two levels is acceptable providing the difference between the 2 levels is not greater than a factor of 10 and providing the response factors of the bracketing calibration standards are within acceptable limits. The response factor of bracketing calibration standards at each level should not differ by more than 20 % (taking the higher response as 100 %).

The acceptance criteria for this parameter is that the Deviation of back-calculated concentration from true concentration is ≤± 20 %.

Example:

(Back calculated concentration – True Concentration)*100/True Concentration

(10-9.8)*100/10 = 2%

Matrix effect

The matrix effect (ME) is evaluated by comparing peak areas of the matrix matched standards (peak area of post-extraction spike) with the corresponding peak areas of standards in solvent. The ME is quantified as the average percent suppression or enhancement in the peak area using the following equation:

$$\mathrm{ME}\ (\%) = \frac{\text{Peak area of matrix matched standard} - \text{peak area of solvent standard}) \times 100}{\text{Peak area of matrix matched standard}}$$

A negative value of ME signifies matrix induced signal suppression, whereas a positive value signifies an enhancement in signal intensity.

Determination of ME is important where a matrix matched standard is used for quantification, however use of procedural standard nullifies importance of matrix effect toa great extent is considered a better practice.

Limit of Quantification

Limit of Quantification (LOQ) is the lowest spike level meeting the identification and method performance criteria for recovery and precision. The LOQ should be less than or equal to MRL.

Specificity

Specificity corresponds to interfering signal of the target analyte in matrix blank or procedural blank. Ideally the matrix blank or procedural blank should be free from such interfering signal and that defines the specificity of the method. If such signals are present in the matrix blank, it should be less than or equal to 30% of the reporting limit. A specific method will not have any interference from the reagent blank, however matrix blank may have inherent contamination. In such case standard addition method can be adopted or a fresh set of calibration can be prepared using another related matrix blank.

Recovery

Spike recovery needs to determined at three different spike concentration, usually at half the MRL value, at MRL value, and at double the MRL value. The average recovery value of all the spike level tested should fall within 70 to 120%. However, recovery outside this range is acceptable when the repeatability relative standard deviation and reproducibility relative standard deviation is less than or equal to 20%.

Precision

Precision indicates the repeatability and reproducibility relative standard deviations of the analytical method. Repeatability RSD_r for each spike level tested should be less than or equal to 20%. Whereas, within-laboratory reproducibility RSD_{wR}, derived from on-going method validation/verification should be less than or equal to 20%. RSD_{wR} value are calculated from the recovery studies carried out on different days by different analysts.

Ion ratio

Ion ratio is an important criteria for quantitative analysis using mass spectrometers in multiple reaction monitoring mode. Percentage ion ratio is determined by the area ratio of qualifier and quantifier ion. The ion ratio of an analyte in the sample should fall within ±30 % of the average ion ratio of all the calibration levels.

References for further reading

1. Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed: Document Nº SANTE/12682/2019.
2. Commission Implementing Regulation (EU) 2021/808 of 22 March 2021
   on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC

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