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The importance of validation methods to facilitate the work of commodity laboratories †

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Abstract: Globalization has increased the trade flow of regional products between countries, making necessary controls to guarantee the safety and authenticity of exported and imported food products. The ISO international certifications recommend traditional analytical chemistry methods to ensure the quality of analytical processes. In the last years, fast automatized methods were developed to approach new analytical problems (there are no recommended methods for determining all food components or toxins), ensure constant performance over time, and avoid the laborious traditional methods requiring professional personnel who know how to apply them. The purposes of validating a chemical analysis method are to prove the adequacy of the procedure in question; document the operator's competence to conduct the work (by verifying the quality parameters obtained through appropriate procedures); provide sufficient data to define the control limits helpful in verifying compliance with the quality parameters during daily work. The validation process must guarantee that the sensitivity of the new tests is equivalent to that of the official methods, their application to the matrix of interest, reliability, and cost-effectiveness. The present work examines the methods used to validate new analytical tests and the statistical approaches used to achieve the goal.

Keywords: Validation; Analytical methods; Metrological approach

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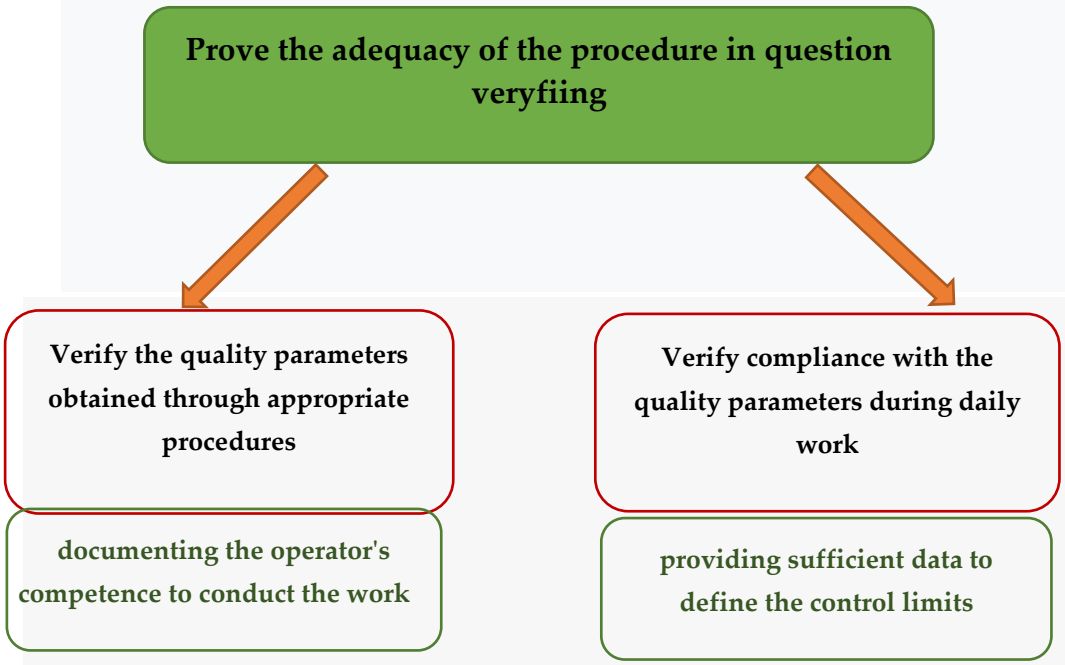
1. Introduction

Today, consumers require healthy, nutritious, tasty, traceable, authentic, safe, ethical, sustainable, and environment-friendly foods [1]. The information's storage in a virtual open space (decentralized public book) and the rigorous testing of the food products by validated analytical methods allow for verification of food quality. Many analytical platforms give targeted and non-targeted information about food components (foodome) related to different "omics" methodologies (i.e., genomics, metabolomics, proteomics, isotopolomic, and metallomics) [2]. The ISO international certifications recognize some traditional analyses as valuable methods to determine food components and toxins [3]. A robust validation procedure is required when non-certified methods are used or changes are made to official methods.

2. The analytical validation process

The analytical food validations can involve Proficiency Testings (PTs), Interlaboratory, and Round Robin trials or employ Reference Materials (RMs) tested for homogeneity and stability with certified values for the properties under study [1]. The method of analysis applied by the laboratory must have stable performance over time to guarantee the comparability of the results, therefore their "quality". The UNI CEI EN ISO/IEC 17025: 2017 standard and related sections specify the measurement validation and reliability criterion (Figure 1) [4].

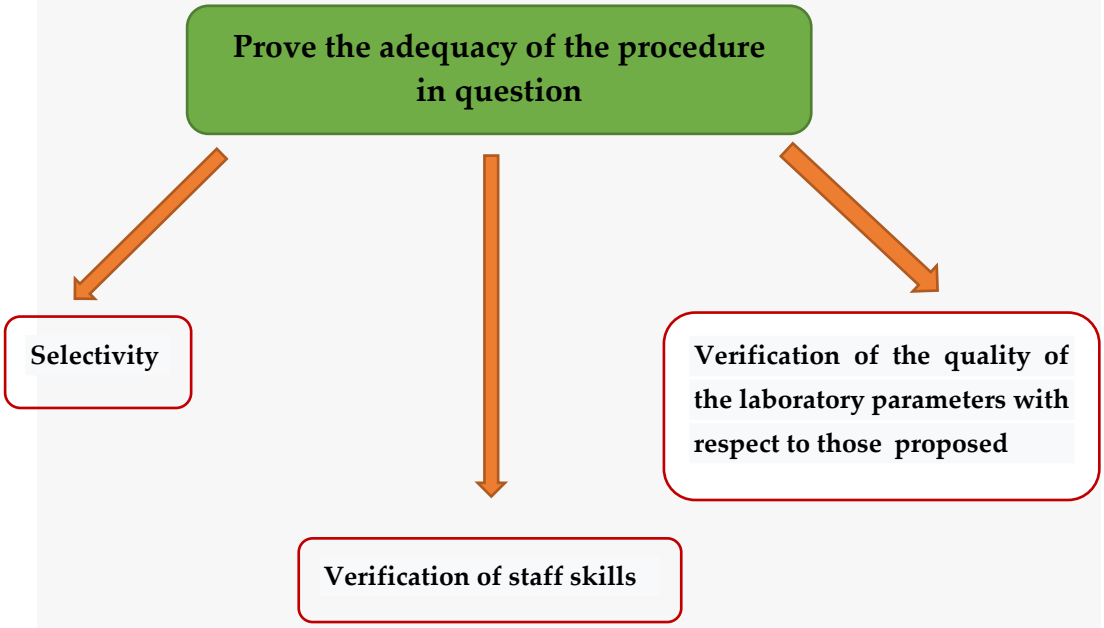
Figure1. Goals of a primary validation process



Two types of validation processes (namely primary and secondary) can be used. As stated in the ISO 16140: 2003 standard, the primary validation process defines the operative limits and performance of a new or standardized method not adequately characterized or a standardized method modified (Figure 2) [5]. It is carried out by the laboratory that developed the method [6]. The validation process of an analytical method involves the consideration of some variables (i.e., appropriate identification; scope; description of the type of object to be tested or calibrated; parameters or quantities and measuring ranges to be determined; equipment, including technical performance requirements; reference samples and required reference materials; environmental conditions and required stabilization period; description of the procedure, including affixing identification marks, handling, transport, storage, and preparation of the objects to be tested; checks to be carried out before starting activities; verification of the proper

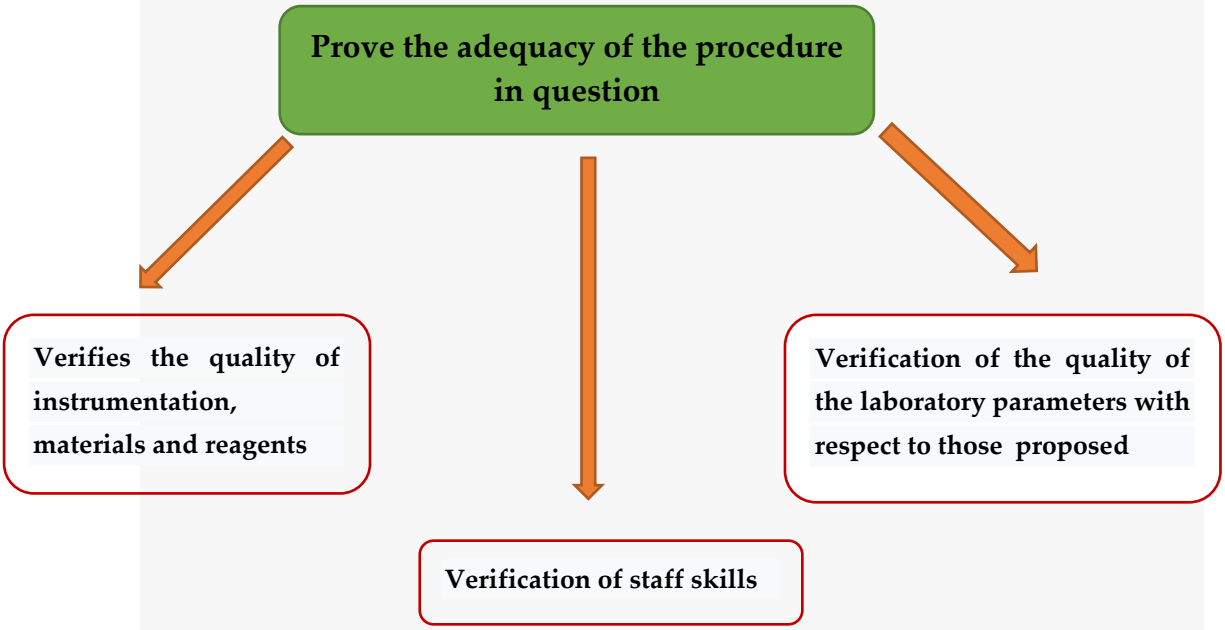
functioning of the equipment and, if required, calibration and fine-tuning before use; methods of recording observations and results; all measures of standard deviation; data to be recorded and the methods of analysis and presentation; uncertainty or uncertainty estimation procedures)[7]. The performance characteristics evaluated are selectivity, specificity, accuracy, the limit of detection (LOD), the limit of quantitation (LOQ), sensitivity, robustness, recovery, and measurement uncertainty (associated with the analytical data).

Figure 2. Goals of a primary validation process



The secondary validation process must be carried out by the laboratories that acquire a method developed elsewhere to verify its ability to apply the method in question with performances not lower than those declared by the validation protocol (Figure 3) [6].

Figure 3. The secondary validation process goals



3. The standard operating procedure (SOP)

The SOP is an operational guide to the validation procedure. It defines the aims and applications of the method, the quality parameters to be evaluated, the experiments to be performed, the verification of the specifications of the tools available, and the description of the necessary quality of standards and reagents for the execution of pre-validation experiments, the revision, if necessary, of the quality parameters and the criteria for acceptance of the results, the execution of all necessary experiments (intra-laboratory and possibly inter-laboratory), the revalidation criteria, the type and frequency of the tests to verify the qualitative suitability of the analytical system and the validation report characteristics (Figure 4) [8].

Figure 4. Typical scheme of validation SOP.

- ✓ Revisions and modifications (authorized by the manager)
- ✓ Title
- ✓ Purpose (type and species of analyte, matrix, range, technique, sample size)
- ✓ Warnings and Precautions
- ✓ Definitions (of any uncommon term)
- ✓ Principle (preferably as a flow-chart)
- ✓ Reagents and materials (including toxicology, purity, storage, labeling, etc.)
- ✓ Instrumentation (typical type and minimum performance, environmental conditions, etc.)
- ✓ Sampling (sample storage, pretreatments, calibration)
- ✓ Quality control (method parameters, type and frequency of controls)
- ✓ Rejection criteria
- ✓ Procedure (including points where quality control is performed)
- ✓ Calculations
- ✓ Expression of results (including rounding, uncertainty, confidence level)
- ✓ Operator
- ✓ Normative references (useful as a theoretical background of the method)
- ✓ Signature of the service manager

4. The quality parameters

The validation procedures depend on the type of analysis to be performed. Given that the methods of analysis can be of a qualitative type, i.e., identify the presence/absence of a specific substance in an analysis sample, or, of a quantitative type, i.e., make a comparison of the quantity of substance present with a reference, to ascertain whether the same is above the legal limit, the analytical data produced must be accompanied by appropriate parameters that indicate its quality. In this regard, following the provisions of the international guidelines (IUPAC Technical Report 2002)[9] and as indicated in the ISO 17025 standard [7]. The accuracy (accuracy and precision), dynamic and linear range, selectivity/specificity, detection limit, quantification, robustness, and recovery must be verified if a quantitative analytical method must be validated. Instead, in validating a qualitative analytical method, the selectivity/specificity, detection limit, and robustness must be subjected to control [7].

4.1 Selectivity

Selectivity defines the ability of an analytical method to respond to the processed analyte rather than interferers or other components. It can be evaluated by analyzing real samples and, if possible, reference materials (having a composition similar to that of real samples) with the method under consideration and with another independent method. Selectivity can be determined by analyzing at least once samples and reference materials using the method under consideration and employing a method based on an independent physical principle. The acceptability of the estimated result must be assessed based on pre-established criteria. The most easily adopted criterion is implicitly linked to the level of confidence chosen to perform the statistical tests [10].

4.2 Working Range

The working range depends on the sample preparation and the analytical procedure used.

4.2.1 Response

4.2.1.1 Linear Response

A linear relationship between analyte concentration and response confirms the procedure's suitability. Statistical methods confirm the test results' reliability (g.e., the value of regression line close to 1, obtained with the method of least squares).

4.2.1.2 Non-linear Response

In these cases, a model or function defines the relationship between the analytical procedure's response and the concentration. The model's suitability should be assessed using non-linear regression analysis, and analytical procedure reliability should be assessed across a given working range to find values proportional to the theoretical or known sample values.

4.2.2 Lower range limits

4.2.2.1 The lover detection limit (LOD),

LOD, or minimum detectable quantity, is the concentration of analyte that produces a signal significantly different from the blank. It can be obtained from standard deviations (σS) through the calibration line and is expressed in concentration units.

$$LOD = 3\sigma S$$

4.2.2.2 The lover quantification limit (LOQ)

LOQ, or minimum detectable quantity, is the minimum concentration of analyte that can be detected [11]

$$LOQ = 10\sigma S$$

4.2.2.3 The dynamic and linear range

The range is the concentration range explored during the measurements.
The dynamic range is the concentration interval in which the signal varies with the concentration: the lower and upper limits of the dynamic range correspond, respectively, to the detection limit and to the highest concentration at which an increase in concentration still produces an increase in signal.
The linear range expresses the concentration range in which the signal varies linearly with the concentration.
The construction of the calibration diagram implies the adoption of a regression method. The ordinary linear least-squares regression method is the most generally adopted method [10]. Conditions necessary to be able to perform an ordinary linear least squares regression:

- the experimental errors associated with the independent variable (concentration, quantity) must be negligible compared to those associated with the dependent variable (signal);
- the errors associated with the dependent variable must be normally distributed;
- the analytical system must be homoskedastic, i.e., the precision must not change significantly as the concentration varies;
- the signal must be a linear function of the concentration. It is also advisable to check for outliers.

Measures can be taken if:

- experimental error affects dependent and independent variables (specific regression methods must be used);
- errors associated with the dependent variable are not normally distributed. In this case, more robust regression methods must be used (e.g., least median of squares);
- the system is heteroskedastic (precision varies significantly with concentration). In this case, it is necessary to transform the data or use weight regression methods;

- the signal is not a linear function of the concentration. In this case, the explored concentration interval must be reduced or, after dividing the interval into sub-intervals, a linear regression must be performed in each sub-interval or to non-linear regression methods.

The statistical tests available are numerous. For instance: the graphical analysis of the regression residuals to define the linearity; the Shapiro-Wilk test to detect the normality; the Dixon and Huber test to detect anomalous values.

4.3 Accuracy

Accuracy is the goodness of the agreement between the average value obtained from an adequately numerous series of results and the accepted reference value. The accuracy can be assessed by analyzing one or more certified reference materials. These must have a composition as similar as possible to the samples under examination. Alternatively, the accuracy can be evaluated by comparing the results obtained by analyzing a series of samples (standard or real) with the method to be validated and with a reference method [10-12]. The T-test can be used to evaluate the difference between the means.

$$\text{Accuracy} = \frac{|\bar{X}_{\text{reference } test} - \bar{X}|}{\sqrt{S_r^2 + U^2_{\text{reference test}}}} \leq t_{p\nu}$$

$\bar{X}_{\text{Voholard } test}$ = mean concentrations obtained with the reference test

$\bar{X}$ = mean concentrations obtained with the new method

S= Standard deviation

U = uncertainty

t = *t* student

4.4 Precision

Precision estimates the agreement between the results of subsequent measurements of the same measure. Repeatability and reproducibility are two types of precision.

Repeatability is the goodness of the agreement between the results of subsequent measurements conducted under the same measurement conditions. The repeatability limit (r) is the maximum value, predictable at a certain confidence level, of the absolute difference between two results obtained under repeatability conditions. The results are suspicious if the difference between the two results is more significant than r.

$$r = t_{1-\frac{\alpha}{2},\nu} * \sqrt{2} * \sigma_r$$

where t1-α / 2, ν is Student's t (2-tailed) for the desired level of confidence and ν = (N-1) degrees of freedom. σr is the standard deviation of repeatability [13].

Reproducibility is the goodness of the agreement between the results of subsequent measurements of the same sample by measuring conducted under non-homogeneous measurement conditions. The reproducibility limit (R) is the maximum value, predictable at a certain confidence level, of the absolute difference between two results obtained under reproducibility conditions. The results are suspicious if the difference between the two results is more significant than R.

The Shapiro-Wilk test can detect the normality of distribution; the Dixon and Huber test finds anomalous values [13].

4.6 Uncertainty

The total uncertainty is a parameter that evaluates the random error sources attributed to the measurand, including systematic error dispersion. It is calculated by estimating the errors associated with the various stages of the analysis, e.g. pre-analytical effects, homogenization, pipetting, weighing, extraction, injection, derivatization, recovery, and calibration curves. (ISO 25, Guide to the Expression of Uncertainty in Measurement ISO, Geneva, 1993).

Currently, with measurement uncertainty we mean the expanded uncertainty, U, obtained by multiplying the combined uncertainty, u, by an appropriate coverage factor, k.

$$U = K*u$$

The coverage factor allows us to associate a confidence level with the ± U interval.

Type A and B uncertainties (U) are determined following the EURACHEM/CITAC guide (EMEA/CHMP/EWP/531305/2008) [14].

Type A uncertainties are associated with method repeatability. They are calculated as follows

$$U \text{ Type A} = \sqrt{\frac{\text{variance}}{\text{Degrees of freedom}}}$$

Type B is defined with a metrology approach. They are related to the standard preparation, the calibration curve, and the apparatus used to perform the analytical method.

U(a) is associated with an analytical balance. It is obtained considering a certificate of calibration, stability, and repeatability;

U(p) is associated with the volume of the pipettes. It is obtained considering a certificate of repeatability and calibration;

U(mr) is associated with the standard used to make the calibration curve;

U(ct) is associated with the calibration curve. It is obtained by measuring the standard in triplicate at three concentrations [10-11].

$$U(ct)\ S\frac{x/y}{b} * \sqrt{1/n + 1/m}$$

S = standard deviation of the residual
n = points used for the calibration line
m = readings taken for each sample

4.7 Recovery

Recovery is the fraction of analyte present or added to the portion of material under test, extracted, and measured. It can be assessed by analyzing a certified reference material, fortifying a white, fortifying a matrix containing the analyte, and comparing the results obtained with a standard method. The recovery depends on the analyte concentration. According to what is specified by the AOAC manual for the Peer Verified Methods program, as the analyte concentration decreases, obtaining recoveries that are increasingly different from 100% is reasonable. A recovery other than 100% indicates the presence of systematic errors [15].

4.8 Robustness

Robustness is the ability of a method to remain unaffected when slight variations are applied. The procedure compares the effects of changes in the influencing factors to the result of the analysis. In the case of a limited number of factors (at most three), the robustness can be evaluated by comparing the average results obtained before and after their arbitrary variation. The factor levels to be examined derive from the specifications of the method in question, detailed in the validation SOP. In the case of significant influence variables, the Youden method can be used. These demonstrated that 8 measurements are sufficient to evaluate the effect of the deliberately imposed variations on 7 influencing factors. The method implies the choice, for each factor, of two levels, higher and, respectively, lower than the standard value of each of the critical factors. By indicating with a +, b +, c +, d +, e +, f +, g + the high levels and with a-, b-, c-, d-, e-, f-, g- the low levels, it is possible to design 8 measurements, one for each combination, of factors [16].

5. Conclusion

Nowadays, great importance is attributed to measuring food quality. The new analytical methods and modified official methods of analysis must be validated. This work analyzes the different validation approaches and statistical tests used to confirm the reliability of the results obtained in food analyses. The validation of an analytical process must analyze the analytical procedures, apparatus, data collection systems, and documentation of the laboratory operations. The instruments' condition should be

confirmed periodically to ensure their functionality. The increase in the analytical testing skills, the harmonization of the research methodologies, the share of the experiences, best practices, networks, the transmit the knowledges through databases and validated analytical methods are fundamental to assure the employment of the legislation about consumer protection.

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