Method Validation of Chemical Analytical Methods of Cannabis and Cannabis-derived Products

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Cannabis sativa is a plant that appears to have great medicinal value. Recent research has identified at least 554 compounds in *C. sativa* plants, among them 113 phytocannabinoids and 120 terpenes.[1] The most prominent cannabinoid is the phytocannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC). Cannabidiol (CBD) is another major constituent of the plant often prevalent in the mainstream media after the signing of the 2018 Farm Bill in relation to hemp. State acceptance of its consumption for both medical and adult-recreational uses is contingent upon accurate, reliable testing for safety and potency. One of the challenges within this environment is that there are no readily available recognized compendial methods to transform the testing from laboratory-developed methods to validated laboratory methods for general laboratory use. Until such a time that recognized compendial methods are available in the marketplace, it is important to validate methods in the laboratory to demonstrate fitness for intended use.

States often require testing for many constituents of cannabis and cannabis-derived products. These include cannabinoids and terpenoids that are defined by genetics and influenced by environmental factors. As the chemical constituents of cannabis can vary dramatically, accurate analytical data on the chemical content and strength of the product is required for its safe and effective use. Information on the content of potential contaminants also is needed to determine suitability for use. In addition, the analytical characterization of physical factors can play a particularly important role in many different formulations of cannabis-derived products. In many state-regulated medicinal cannabis programs, products are analyzed for cannabinoid and terpene profiles, and for the absence of heavy metals, pesticides, bacteria, molds, and fungal toxins. In the current environment, laboratories find that a method is needed as a published standard.

ISO/IEC 17025:2017, *General requirements for the competence of testing and calibration laboratories*, requires that validation be performed for non-standard methods, laboratory-developed methods, or standard methods used outside of their scope. Furthermore, "performance characteristics of validated methods, as assessed for the intended use, shall be relevant to the customers' needs and consistent with specified requirements."[2] As stated above, because the current methods used for the cannabinoids and terpenes are laboratory-developed, they require validation. Even the U.S. EPA methods frequently used to characterize pesticides or heavy metals are commonly modified for cannabis, so they also require validation.

A related note in ISO/IEC 17025 states: "The techniques used for method validation can be one of, or a combination of, the following: a) calibration or evaluation of bias and precision using reference standards or reference materials; b) systematic assessment of the factors influencing the result; c) testing method robustness through variation of controlled parameters, such as incubator temperature, volume dispensed; d) comparison of results achieved with other validated methods; e) interlaboratory comparisons; f) evaluation of measurement uncertainty of the results based on an understanding of the theoretical principles of the method and practical experience of the performance of the sampling or test method."[3]

As the standard is written to reflect a wide variety of laboratories, these notes and requirements appear to provide little direction on what is specifically needed for the validation of methods related to the chemical analysis of cannabis and cannabis-derived products. As the medical use of cannabis becomes more prevalent and state accepted, a pharmaceutical approach to method validation would be most appropriate. Adopting a pharmaceutical approach

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[1]Aizpurua-Olaizola O, Soydaner U, Öztürk E, Schibano D, Simsir Y, Navarro P, Etxebarria N, Usobiaga A (February 2016). "*Evolution of the Cannabinoid and Terpene Content during the Growth of Cannabis sativa Plants from Different Chemotypes*". Journal of Natural Products. 79 (2): 324–31.
[2]ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories*, ISO Geneva, 2017.
[3]ISO/IEC 17025, *General requirements for the competence of testing and calibration laboratories,* ISO Geneva, 2017.

will allow laboratories the use of a readily accepted and standardized approach to method validation for chemical analysis.

Method development is a staged process as identified in Figure 1, ISO V model adopted for analytical method development. The left side of the V identifies the objectives of the development. The right side of the V indicates the processes and procedures needed to document that the objectives have been achieved. Method development and validation is always a balance between costs, risks, and technical issues. The laboratory should do its best within the constraints imposed, taking into account customer and regulatory requirements, existing experience of the method, available quality controls, and the need for commutability with similar methods already in use.

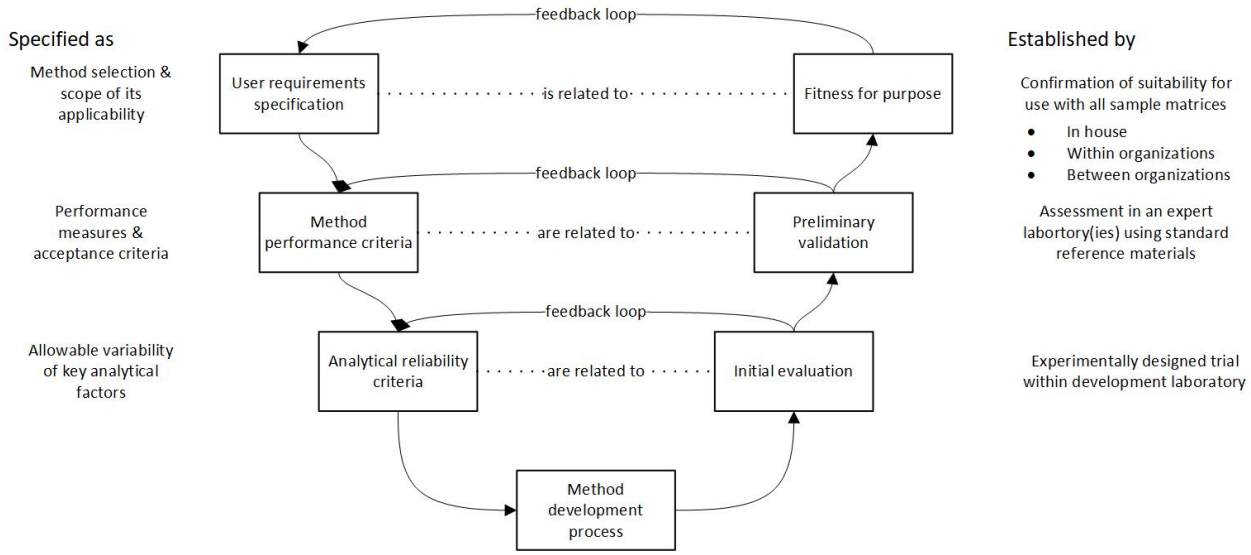

Figure 1. ISO V model adapted for analytical method development[4]

Validation has been defined as the confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled.[5] The United States Pharmacopeia (USP) states, "Validation of an analytical method is the process by which it is established, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications."[6] The U.S. Food and Drug Administration (FDA) defines analytical method validation as the process of demonstrating that an analytical method is suitable for its intended purpose.[7] What is not identified in these definitions is that the actual method validation package should be the result of a well-organized, well-planned, and methodically implemented validation process. The validation package generated by the laboratory should demonstrate how the acceptance criteria were met, including references to raw data. The validation package and report should be reviewed and approved by appropriately qualified personnel.

[4]Burgess, Christopher, *Valid Analytical Methods and Procedures,* Royal Society of Chemistry, 2001.
[5]ISO 9000, *Quality management systems - Fundamentals and vocabulary,* ISO Geneva, 2015.
[6]USP40- NF35, General Chapter 1225, *Validation of Compendial Methods,* Rockville, MD, 2017.
[7]USFDA, *Analytical Methods and Methods Validation for Drugs and Biologics Guidance for Industry*. Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, MD, 2015.

Extent of Validation

When using method validation guidance from organizations such as the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH),[8] the FDA,[9] or USP,[10] it is important to note that there is not one type of validation that covers all methods. Different analytical performance characteristics are required for the different classifications of analytical methods.

The analytical methods that need to be validated are classified per ICH[11] as follows:

- Identification tests: to ensure identity of an analyte.
- Quantitative test for impurities: to accurately and quantitatively reflect the purity of a sample.
- Limit test for impurities: to reflect purity characteristics of the sample.
- Assay of drug substance and drug products: to measure accurately and quantitatively the analyte present in the sample.

Typical analytical performance characteristics to be considered when using the ICH approach for validation are listed in Table 1.

Table 1. ICH[12] Validation Characteristics (Adapted)

Analytical Performance Characteristic	Identification	Testing for Impurities		Assay: Content/Potency
		Quantitation	Limit	
Accuracy	−	+	−	+
Precision				
… Repeatability	−	+	−	+
… Intermediate precision	−	+	−	+
Specificity	+	+	+	+
Detection limit	−	−	+	−
Quantitation limit	−	+	−	−
Linearity	−	+	−	+
Range	−	+	−	+

- Indicates this characteristic need not be considered
\+ Indicates this characteristic needs to be considered

USP prescribes a similar, yet slightly different approach.[13] Test requirements vary from exceedingly rigorous analytical determinations to subjective evaluation of qualities. According to USP, considering this broad variety, different test methods require different validation schemes. Different analytical data are needed to determine the fitness for purpose of the analytical method validated. USP categories of methods for validation are as follows:[14]

- Category I Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.
- Category II Analytical methods for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These methods include quantitative assays and limit tests.

[8]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, incorporated 2005.
[9]USFDA, *Analytical Methods and Methods Validation for Drugs and Biologics Guidance for Industry*. Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, MD, 2015.
[10]USP40- NF35, General Chapter 1225, *Validation of Compendial Methods,* Rockville, MD, 2017.
[11]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, incorporated 2005.
[12]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, incorporated 2005.
[13]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[14]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.

- Category III Analytical methods for determination of performance characteristics (e.g., dissolution, drug release, and others).
- Category IV Identification tests.

Table 2: USP[15] Validation Characteristics (Adapted)

Analytical Performance Characteristic	Category 1	Category II		Category III	Category IV
		Quantitative	Limit		
Accuracy	+	+	*	*	
Precision	+	+		+	
Specificity	+	+	+	*	+
Limit of Detection			+	*	
Limit of Quantitation		+		*	
Linearity	+	+		*	
Range	+	+	*	*	
Ruggedness	+	+		+	

\+ Indicates this characteristic needs to be considered
* Indicates this characteristic may be considered depending on nature of tests

Characteristics of Validation

Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value.[16] This is sometimes termed trueness. It is recommended that accuracy be determined using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range.[17]

Accuracy is measured as the percent of analyte recovered by the assay.

The recovery can be determined by the equation:

$$\text{Recovery} = \frac{\text{Analytical Result}}{\text{True Value}} \times 100\%$$

The recovery should be in the range of the control limit specified in the validation method.

The following method can be applied for calculating the upper control limit (UCL) and lower control limit (LCL). The method involves the moving range, which is defined as the absolute difference between two consecutive measurements ($|x_i - x_{i-1}|$).

The moving range is averaged ($\overline{MR}$) and used in the following formulae:[18]

$$UCL = \bar{x} + 3\frac{\overline{MR}}{d_2} \quad \text{and} \quad LCL = \bar{x} - 3\frac{\overline{MR}}{d_2}$$

Where, x_i is the individual analytical result, $\bar{x}$ is the sample mean, and d_2 is a constant based on the number of observations associated with the moving range calculation. Where n = 2 (two consecutive measurements), as here, $d_2 = 1.128$

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[15]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[16]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[17]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[18]USP38-NF33, General Chapter 1010, ANALYTICAL DATA—INTERPRETATION AND TREATMENT, Rockville, MD, 2017.

Note this is not meant to be an all-inclusive primer on approaches for accuracy or any other characteristics. Other statistical approaches may be used.

Precision

The precision of an analytical method is the degree of agreement among individual test results when the method is repeated on six or more samplings of a homogeneous sample.[19] The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.

For example, relative standard deviation (RSD) is determined by the equation:

$$RSD(\%) = \frac{100}{\bar{x}}\left[\frac{\sum_{n-1}^{n}(x_i-\bar{x})^2}{n-1}\right]^{\frac{1}{2}}$$

Where x_i is an individual measurement in a set of n measurement and is the arithmetic mean of the set. In pharmaceutical method validation, the RSD should not be more than 2%. Intermediate precision is the results from within lab variations due to random events such as different days, different analysts, different equipment, etc.[20]

It is helpful to evaluate either the standard deviation or the relative standard deviation for each type of precision determined.

Repeatability

Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment.[21] Repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).[22]

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).[23] Reproducibility is not always observed for single-laboratory validation. However, it is beneficial when an analytical method is standardized or is going to be used in more than one laboratory.

Selectivity and Specificity

Specificity is the ability to measure the analyte of interest in the presence of other components that may be expected to be present in the sample matrix, such as impurities, degradation products and matrix components. It must be demonstrated that the analytical method is unaffected by the presence of spiked materials, because real-life samples are usually mixtures of many compounds and the analytical method must therefore be selective toward the analyte of interest.

IUPAC defines selectivity as the extent to which other substances interfere with the determination of a substance according to a given procedure.[24] The larger the interference, the less selective the procedure. As the definition implies, methods can be selective to different extents. If a given method is 100% selective, it is said to be specific. Analytical techniques are almost never generally specific, or it is nearly impossible to prove that. However,

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[19]Guideline on bioanalytical method validation, European Medicines Agency, London, UK, 2011.
[20]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[21]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[22]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[23]Validation of Analytical Procedures SC III F, British Pharmacopeia, British Pharmacopeia Commission, 2013.
[24]IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). on-line corrected version: http://goldbook.iupac.org (2006); accessed February 20, 2019.

analytical methods can be specific within their scope of application, i.e., for a given analyte in a given matrix in a given concentration range.

Note that terminology regarding selectivity and specificity is not used unanimously in validation guidelines. FDA, AOAC International, and Eurachem guidelines use the terms suggested by IUPAC, while ICH uses the term specificity to denote selectivity.

In the case of identification tests, the method should be able to discriminate between compounds of closely related structures that are likely to be present. Similarly, in the case of assay and impurity tests by chromatographic procedures, for example, specificity can be demonstrated by the resolution of the two components that elute closest to each other.[25]

It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte. In this case, a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.

Linearity

Linearity is the ability of the method to elicit results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range.[26] Linearity should be established initially by visual examination of a plot of signals as a function of analyte concentration. If there appears to be a linear relationship, linearity should be established by appropriate statistical methods. Data from the regression line provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, and the slope of the regression line should be included in the validation data package.

It is recommended to have a minimum of five concentration levels, along with certain minimum specified ranges. For assays, the minimum specified range is from 80% -120% of the target concentration.[27]

Regression line, $y = ax + b$

Where, a is the slope of regression line and b is the y- intercept.

Here, x may represent analyte concentration and y may represent the signal responses.

Correlation Coefficient:

$$r = \frac{\sum n\ (x_i - \bar{x}\ )(y_i - \bar{x})}{[\sum x_i - \bar{y}\ )(x_i - \bar{y})]^{1/2}}$$

Where x_i is an individual measurement in a set of n measurement and $\bar{x}$ is the arithmetic mean of the set, y_i is an individual measurement in a set of n measurement and $\bar{y}$ is the arithmetic mean of the set.

Detection Limit and Quantitation Limit

The detection limit is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. The quantitation limit is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the analytical procedures.[28] Some of the approaches to determine the detection limit and quantitation limit are: [29]

Signal-to-Noise

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte

[25]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[26]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[27]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[28]USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[29]ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.

with those of blank samples. This can be used to establish the minimum concentration at which the analyte can be reliably detected and in the determination of the detection limit and reliably quantified for the determination of quantitation limit. A signal-to-noise ratio between 3:1 or 2:1 is generally considered acceptable for estimating the detection limit and a typical signal-to-noise ratio is 10:1 is considered acceptable for establishing the quantitation limit.

Standard Deviation of the Response and the Slope

The detection limit (DL) can be expressed as:
$DL = 3.3\sigma / s$

The quantitation limit (QL) can be expressed as:
$QL = 10\sigma / s$

Where, σ is standard deviation of the response and s is slope of the linearity curve.

The method used for determining the DL and the QL should be presented in the validation package. If DL and QL are determined based on visual evaluation or based on signal-to-noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification.

There are many other approaches to estimating both the detection limit and the quantitation limit, each using different statistical assumptions and method considerations.

Range

The range of an analytical procedure is the interval between the upper and lower levels of analyte that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the analytical procedure as written. The range is normally expressed in the same units as the test results obtained by the analytical procedure.[30]

The following minimum specified ranges should be considered:[31]

- For assay of a drug substance (or a drug product) the range should be from 80% to 120% of the test concentration.
- For determination of an impurity the range should be from 50% to 120% of the acceptance criterion.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters and provides an indication of the procedure's suitability during normal usage. Robustness may be determined during development of the analytical procedure.[32]

In the case of liquid chromatography, examples of typical variations are:

- Influence of variations of pH in a mobile phase;
- Influence of variations in mobile phase composition;
- Different columns (different lots and/or suppliers);
- Temperature;
- Flow rate.

In the case of gas-chromatography, examples of typical variations are:

- Different columns (different lots and/or suppliers);

[30] USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.
[31] ICH Q2(R1) *Validation of Analytical Methods: Text and Methodology,* Geneva, 2005.
[32] USP40-NF35, General Chapter 1225, *Validation of Compendial Methods*, Rockville, MD, 2017.

- Temperature;
- Flow rate.

Quality Control Types Used in Validations

The use of quality controls in validation guarantees that methods of analysis are fit for their intended purpose. In this respect, both systematic errors, leading to bias, as well as random errors, leading to imprecision, are monitored. To be able to monitor these errors, they should remain constant. Within the laboratory, such constant conditions are typically achieved in one analytical run. Thus, monitoring the precision as an objective of validation does not concern reproducibility or interlaboratory precision, but only repeatability or intralaboratory precision.

Blanks

Use of various types of blanks enables assessment of how much of the measured signal is attributable to the analyte and how much is to other causes. Types of blank available are:

Reagent blanks: Reagents used during the analytical process (including solvents used for extraction or dissolution) are analyzed to determine whether they contribute to the measurement signal.

Sample blanks: These are essentially sample matrices with no analyte present, e.g., *Cannabis sativa* without pesticides present. Sample blanks may be difficult to obtain but such materials are necessary to give a realistic estimate of interferences that would be encountered in the analysis of test samples.

Routine test samples

Routine test samples are useful because of the information they provide on precision, interferences, and other aspects of the test that could realistically be encountered in day-to-day work. If the analyte content of a test material is accurately known, it can be used to assess measurement bias.

Spiked materials or solutions

Spiked materials or solutions are materials or solutions to which the analyte(s) of interest have been added. These materials or solutions may already contain the analyte of interest, so care is needed to ensure the spiking does not lead to analyte levels outside the working range of the method. Spiking with a known amount of analyte enables the increase in response to the analyte to be measured and calculated in terms of the amount added, even though the absolute amounts of analyte present before and after addition of the spike are not known. Note that most methods of spiking add the analyte in such a way that it will not be as closely bound to the sample matrix as it would be if it was present naturally. Therefore, bias estimates obtained by spiking can be expected to be unrealistic.

Spiking does not necessarily have to be restricted to the analyte of interest. It could include anything added to the sample to gauge the effect of the addition. For example, the sample could be spiked with varying amounts of a particular interference in order to judge the concentration of the interferent at which determination of the analyte is adversely affected.

Reference Materials

It is important to distinguish between reference materials (RMs) and certified reference materials (CRMs)[33] because of the significant difference in how they can be used in method validation. RMs can be any material used as a basis

[33]ISO Guide 30, *Reference materials – Selected terms and definitions*, ISO Geneva, 2015.

for reference and could include laboratory prepared standards of known purity. The property or analyte of interest needs to be stable and homogenous, but the material does not need to have the high degree of characterization, metrological traceability, uncertainty, and documentation associated with CRMs.

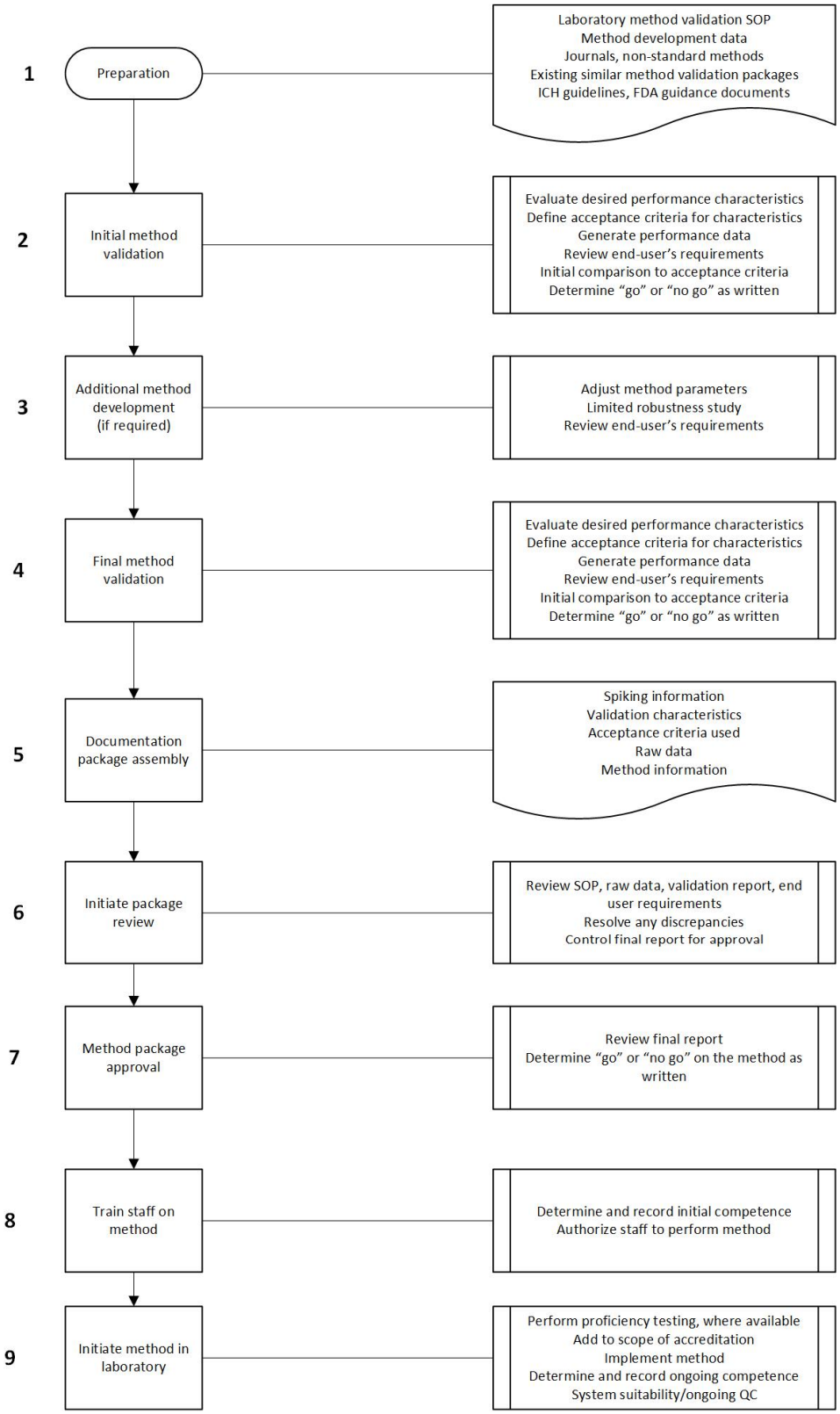
The characterization of the parameter of interest in a CRM is more strictly controlled than for an RM, and, in addition, the characterized value is certified with documented metrological traceability and uncertainty. Characterization is normally achieved so that, as far as possible, any bias is reduced or even eliminated.

Assessment of bias for method validation requires a reliable reference standard, preferably a CRM, with the same matrix and analyte concentrations as the test samples.

Validation Process

Below are considerations for a validation process to demonstrate the method meets the needs of the end-user and the requirements of ISO/IEC 17025:2017. This is a general process that identifies the aspects to bring method validation to an acceptable closure. The process flow below identifies considerations of the steps, documents, and sub-processes related to method validation.

Figure 2. Method Validation Simplified Process Flow



Conclusion

Method validation is a key activity in chemical analysis, indispensable for obtaining reliable results. The higher the complexity of the method, the more important and voluminous, as a rule, is validation. Methods related to cannabis testing are notorious for their complexity, on the one hand because of the instrument itself, and on the other hand because cannabis and cannabis-related products are complex samples. Therefore, it is important to demonstrate that methods are working as expected (validation) and the obtained results are reliable. This information is relevant both to the laboratory (to be confident in your results or to make adequate changes in method if the performance is not as expected) and the customer. Method validation is an essential part of good measurement practice because valid data can be produced only when the strengths and weaknesses of a method are understood.