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How Composition Methods Are Developed and Validated

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ABSTRACT: Method validation is a critical prerequisite to performing analytical methods in the laboratory. A given analytical method is validated for a specific matrix or matrices. If the matrix to be tested is not included in the original scope of method validation, a validation must be performed to determine if the method is applicable to that particular matrix. A number of organizations, such as AOAC and ISO, publish peer-reviewed methods for cross-industry matrices, whereas others, such as AOCS and AACC, are focused on specific industry segments (fats/oils and cereal grains). When no validated method is available for the analyte of interest, method development and validation must first be performed to ensure that correct identification and quantification of the analyte are being observed and measured. Development of a new method requires an understanding of the chemistry and properties of the analyte to be tested, as well as the various types of instrumentation currently available. Method development and improvement is a continuous process, as technology advances and new instrumentation and techniques become available. This paper addresses some of the decisions related to method development but will primarily focus on validation as it applies to compositional testing of foods, crops, and commodities, the factors that determine method selection, and how extensive the validation need be.

KEYWORDS: *method validation, method verification, method development, analytical method, analytical testing, laboratory testing, analytical matrices*

INTRODUCTION

Method development is the process undertaken to determine the analytical steps required to identify a specific analyte in a given matrix. There are many different types of analytical methods, and often an analyte can be tested by numerous methods. Choosing the correct method to use is determined by analyte level, the matrix to be tested, and the purpose for the analysis. Method development is a process that the developer rarely has to start without any background information, as there are many existing types of analytical procedures, instrumentation, and approved methods already available to use as a basis for developing a new method. This paper will discuss method development and validation as it is conducted at a specific contract research organization. Although certain aspects, details, and steps may vary between laboratories, the general process will be similar for any laboratory involved in method development and validation.

Method validation is an essential and necessary element in the implementation of any laboratory analytical method. The Association of Official Analytical Chemists (AOAC) defines the validation of a method as follows: The planned and documented procedure establishes its performance characteristics. The performance characteristics or the validation parameters of the method determine the suitability for its intended use. They define what the method can do under optimized conditions of matrix solution, analyte isolation, instrumental settings, and other experimental features. The inclusion of particular validation parameters in a validation protocol depends on the application, the test samples, the goal of the method, and domestic or international guidelines or regulations, as applicable.[1]

Method validation is the last phase of the method development process. Most methods used in the contract laboratory are published, peer-reviewed methods that have been through a validation, but the scope of these methods is typically limited to a single matrix or group of matrices. When a new method is to be used in the laboratory and that method is not changed or modified and the matrix is included in the scope of the method's validation, a full validation is not necessary and therefore not normally performed. For this scenario, an abbreviated validation, or verification, is performed to show that analysts are indeed able to perform the method as written for the applicable matrices, under the laboratory's conditions. Method validations are required when an established method has been modified or the matrices to which the method is going to be applied have not been validated against the method parameters.

The components of a validation vary between industries, application, regulatory classifications, and laboratories, but evaluation of a common group of characteristics is typically included in any method validation.

METHOD DEVELOPMENT

Analytical Method Categories. Analytical methods generally fall into one of two different categories: wet chemistry (bench chemistry) or instrumental analysis. Each category can be further divided into additional types of procedures. Wet chemistry techniques are hands-on procedures and are very empirical in nature. Two examples of some wet chemistry techniques include titrations, which measure the concentration of an analyte on the basis of its reaction with a measured

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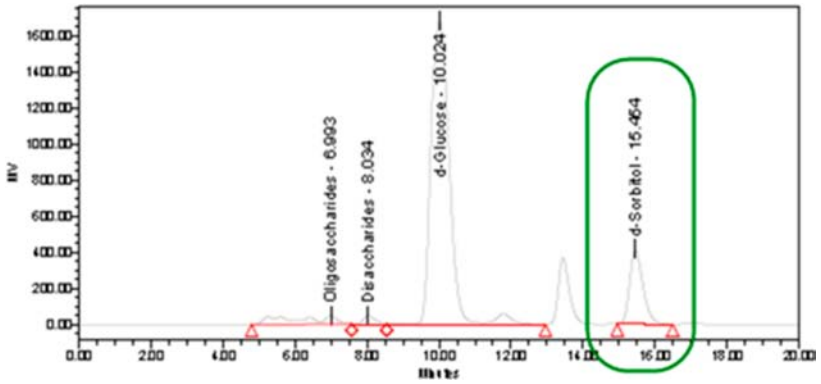

Figure 1. Chromatographic peak of *d*-sorbitol.

amount of a second solution, and gravimetric methods, which determine the quantity of an analyte on the basis of the solid weight remaining after a reaction (e.g., drying, precipitation). The second category of analysis is instrumental. Instrumental methods are typically more sophisticated, automated methods. An instrument used frequently for laboratory analysis is the high-performance liquid chromatograph (HPLC). HPLCs use specialized columns to separate the components of a solution containing the analyte of interest. The various separated compounds are monitored by specific types of detectors (e.g., fluorescent, UV−vis, electrochemical, mass spectroscopy) for the identification and quantification using predetermined detection settings.

Steps of Method Development. The first step in developing a method is a literature search. There are many methods already available through various industry publications, associations, universities, and other research organizations. Examples of these are the OMA (Official Methods of Analysis) from the AOAC,[2] methods from the AOCS,[3] Approved Methods of Analysis from the AACC,[4] and Standards Catalogue from the ISO.[5] Often a method is already available for the analyte of interest, and this can be used as the basis for developing the new method. Sometimes the same analyte may have been examined in other types of matrices, which provides valuable information in the method development process. When no method exists for the analyte, the next step will be to look for an analytical method where a related analyte has properties (physical and/or chemical) similar to the analyte of interest. Development at this stage becomes a matter of experimentation to find the correct analytical parameters, such as pH, extraction solution, mobile phase, type of liquid chromatography column, and retention time, for the method.

METHOD VALIDATION

Once a method has been developed, the details of the method are formally described in a written document and locked to prevent changes to the method. The method protocol is then written. A method validation is a formal process that includes both the written and approved method as well as the protocol. All deviations from the protocol must be approved and documented in the validation report. If, during the course of the validation, significant issues arise, the validation will halt and will return to the developmental stage to resolve the unanticipated issues. Method validations vary in regard to the components of the validation and the terms used to describe those components. Many associations, such as the AOAC,[6] FDA,[7] and FAO,[8] provide guidelines for method validation. There are a key set of elements that are generally accepted as critical for an analytical method validation. These elements are

(1) specificity
(2) accuracy
(3) precision
(4) limit of detection (LOD) and limit of quantification (LOQ)
(5) linearity and range
(6) robustness and ruggedness

Specificity. Specificity is the ability of a method to correctly identify the analyte of interest, and only that analyte, as seen with the *d*-sorbitol peak in Figure 1. There may be other compounds that interfere by either inhibiting or amplifying the detection and quantification of the analyte of interest. The ideal means for identifying a potentially interfering compound is to include a blank matrix in the validation. This is the matrix that is to be tested, but without the analyte of interest. For a genetically engineered crop, this could include testing of the nonengineered variety, where the genetic transformation involves biofortification or elimination of the analyte of interest. For some validations, it is not always possible to obtain a sample of the same matrix, or a similar matrix, without the analyte of interest and, in these cases, reagent blanks may be used as a substitute.

Accuracy. Accuracy is the capability of an analytical method to determine the correct measurement, or exactness, of an analyte concentration. The preferred means for doing this in a validation is to include standard, or certified, reference materials (SRMsor CRMs, respectively). SRMs and CRMs are external samples that are commercially available and have guaranteed levels or reference ranges for the analyte of interest. Ideally, the matrix of the reference sample will be the same, or similar to, the matrix to be validated. When no SRM or CRM is available, the other options are use of previously run check samples, internal control samples, comparison of results to a secondary method(s), or comparison of results to a secondary laboratory (or laboratories). Spiking or fortification studies are also included in the method validation to help in determining accuracy. Although spike recoveries show that the analyte is able to be clearly detected and quantified, they do not confirm the effectiveness of the extraction. This is why it is very important to have a control of the same or similar matrix that contains endogenous levels of the analyte of interest.

Precision. Precision is the ability of an analytical method to produce consistent results. The precision of a method is determined by its repeatability, intermediate precision, and reproducibility. Repeatability is the consistency of results

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Table 1. Intermediate Precision: Matrix Sample Results − Protein, Combustion

sample[a]	instrument[b]	first-person results summary (%)	second-person results summary (%)
matrix 1	A	av, 32.06 SD, 0.22; RSD, 0.69 internal control range, 31.34−32.69	av, 32.01 SD, 0.34; RSD 1.05 internal control range, 31.34−32.69
matrix 2	B	av, 0.30 SD, 0.01; RSD, 2.25 internal control range, 0.29−0.37	av, 0.33 SD, 0.02; RSD, 5.70 internal control range, 0.29−0.37
matrix 3	C	av, 11.32 SD, 0.04; RSD, 0.31 internal control range, 11.18−11.68 CRM spec, 10.63−12.20	av, 11.43 SD, 0.13; RSD, 1.11 internal control range, 11.18−11.68 CRM spec, 10.63−12.20
matrix 4	D	av, 40.75 SD, 0.13; RSD, 0.32 internal control range, 40.20−41.18 CRM spec, 38.42−41.51	av, 40.69 SD, 0.25; RSD, 0.61 internal control range, 40.20−41.18 CRM spec, 38.42−41.51

[a]Matrix 1 refers to a feed sample, matrix 2 to a low-fat salad dressing, matrix 3 to a pet food, and matrix 4 to a dietary supplement. [b]Instruments A, B, and C refer to Leco Nitrogen Analyzer model 601-500-100, and instrument D refers to Leco Nitrogen Analyzer model FP-628.

Table 2. Intermediate Precision: Matrix Results − Vitamin B12, Microbiological

sample[a]	instrument[b]	first-person results summary (μg/100 g)	second-person results summary (μg/100 g)
matrix 1	A	av, 1.27 SD, 0.22; RSD, 6.75 internal control range, 1.22−1.47 SRM spec, 1.17−1.54	av, 1.34 SD, 0.093; RSD, 6.93 internal control range, 1.22−1.47 SRM spec, 1.17−1.54
matrix 2	A	av, 18.9 SD, 1.13; RSD, 5.99 internal control range, 17.6−20.5	av, 19.8 SD, 1.10; RSD, 5.53 internal control range, 17.6−20.5
matrix 3	B	av, 192 SD, 10.0; RSD, 5.21 internal control range, 188−217	av, 202 SD, 9.3; RSD, 4.60 internal control range, 188−217
matrix 4	B	av, 0.495 SD, 0.0397; RSD, 8.02 internal control range, 0.0480−0.551	av, 0.530 SD, 0.0423; RSD, 7.80 internal control range, 0.0480−0.0551

[a]Matrix 1 refers to a frozen dinner, matrix 2 to an animal feed, matrix 3 to a dietary supplement, and matrix 4 to an infant formula. [b]Instruments A and B refer to Schaefer Technologies' Autoturb III.

between runs by the same analyst, using the same instrument with the same conditions. Intermediate precision is the consistency of results of a second analyst who performs the run under the same conditions. The second person's assays may or may not be performed using the same instrument as the first person's. Intermediate precision is the measure of a laboratory to produce consistent intralaboratory results, or consistency between its analysts, using the same method and conditions. Reproducibility provides data regarding interlaboratory consistency or consistency of results between different laboratories. Repeatability, intermediate precision, and reproducibility are all expressed as the percent relative standard deviation (%RSD) between replicate data points. Analytical variables that determine precision include the analyte, method type, and instrumentation used. For example, in Table 1, the protein (nitrogen by combustion) assay has a relatively low %RSD using data from two different analysts on different days, with different instrumentation. Analytes that are subjected to degradation (e.g., water-soluble vitamins) with exposure to heat, oxygen, and light often have a higher %RSD compared to more stable analytes, as seen in Table 2.

The differences in %RSD between Tables 1 and 2 reflect the differences between the analyte and method used for detection and quantification. The protein method is simpler and more automated and has fewer steps, thus limiting the probability of error. The sample is combusted at a very high temperature, where nitrogen is released and then quantified. Nitrogen is then converted to protein using a factor appropriate for the sample type. Levels of vitamin B12 in food are typically low and are tested using a microbiological method. This method requires multiple steps and several dilutions and is dependent on the growth of a microorganism consuming the vitamin in the sample and standards. The standards are plotted using a polynomial curve, and results for each sample are calculated from the curve. Because of the multiple steps required for this

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assay, the probability of error and number and complexity of variables results in a higher overall %RSD for the assay.

Limit of Detection and Limit of Quantification. The LOD is the lowest concentration of an analyte that can be detected for a method. The LOD may be calculated using a 3:1 signal-to-noise ratio. The LOQ is the lowest concentration of an analyte that can be quantified for a method. The LOQ may be calculated using a 10:1 signal-to-noise ratio. The "noise" for data in Table 3, obtained using an HPLC method, was determined by analyzing a sample with low levels of the analyte in multiple runs and calculated as the standard deviation (SD). The signal-to-noise ratio may vary depending on the analytical method used (e.g., ICP, ELISA, microbiological) as all measured readings, or signals, vary in their strength and contain some type of background noise.

Another, more pragmatic, approach is to calculate the LOQ from the lowest standard in the standard curve. This approach requires that the standard curve has a good correlation coefficient.

Linearity and Range. Linearity is an expression of how well the standard points in a regression line correlate. The linearity of the standard curve determines what range is acceptable for use for the method. The term "linearity" is used even when the standard curve is nonlinear (e.g., polynomial, exponential, power, logarithmic curves). In these cases, it is the correlation coefficient of the standard curve's points that determines its "linearity".

Robustness and Ruggedness. Robustness is the ability of a method to produce consistent results under various (normal) conditions, such as the use of different laboratories, different analysts, or different instruments. Ruggedness is the ability of a method to produce consistent results under challenged conditions, those that have been intentionally altered. These changes, such as pH, extraction conditions, or solvent concentrations, are outside the scope of the method's parameters. Ruggedness testing is often performed using Youden's[9] matrix, where eight combinations of seven factors are used to test the ruggedness of an analytical method.

Table 4 provides an example of a ruggedness study for protein, using soy meal as the control sample. Seven "challenge" factors that could potentially affect the result were chosen and assigned values (A, a, B, b, C, c, etc.), and then eight assays were then performed on the different trials (Table 5). Using Youden's method, calculations were then performed on the result data to determine the effect of each factor combination (Table 6). Positive values indicate a result that is higher than results under normal conditions; negative values indicate a result that is lower than results under normal conditions. The values are then plotted on a chart to show their relative significance (Figure 2).

Table 3. LOD and LOQ Results

run	ferulic acid (ppm)	run	ferulic acid (ppm)
1	11.64	6	11.17
2	11.51	7	11.99
3	11.32	8	10.57
4	11.27	9	11.57
5	11.42	10	11.25
	av, 11.371		
	SD, 0.369		
	%RSD, 3.25		
	3 × SD (LOD), 1.11		
	10 × SD (LOQ), 3.69		

Table 4. Ruggedness Challenge Design

factor	value from method	challenge testing
oxygen level (first stage)	(A) high	(a) medium
oxygen level (second stage)	(B) high	(b) medium
sample treatment	(C) ground	(c) unground
sample weight	(D) 0.2 g	(d) 0.4 g
delay after preparation	(E) no delay	(e) 1 h in open air
capsule (weigh boat)	(F) vegetable capsule	(f) aluminum foil
temperature	(G) 900 °C	(g) 800 °C

Table 5. Ruggedness Test Results

trial	factor combinations	protein (%)
T1	A B C D E F G	48.51
T2	A B c D e f g	49.22
T3	A b C d E f g	49.05
T4	A b c d e F G	48.94
T5	a B C d e F g	48.86
T6	a B c d E f G	48.65
T7	a b C D e f G	49.15
T8	a b c D E F g	48.73

Table 6. Calculation of Factor Combination Values

factor A = (T1 + T2 + T3 + T4 − T5 − T6 − T7 − T8)/4 = +0.082, or +0.17%

factor B = (T1 + T2 − T3 − T4 + T5 + T6 − T7 − T8)/4 = −0.155, or −0.32%

factor C = (T1 − T2 + T3 − T4 + T5 − T6 + T7 − T8)/4 = +0.008, or +0.02%

factor D = (T1 + T2 − T3 − T4 − T5 − T6 + T7 + T8)/4 = +0.025, or +0.05%

factor E = (T1 − T2 + T3 − T4 − T5 + T6 − T7 + T8)/4 = −0.309, or −0.63%

factor F = (T1 − T2 − T3 + T4 + T5 − T6 − T7 + T8)/4 = −0.258, or −0.53%

factor G = (T1 − T2 − T3 + T4 − T5 + T6 + T7 − T8)/4 = −0.155, or −0.32%

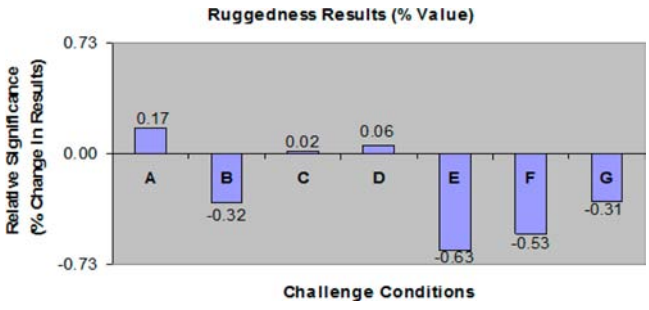

Figure 2. Relative impact of ruggedness variables.

Challenges of Method Development and Validation for Compositional Analysis of Crops. One of the biggest challenges in both method development and validation is the availability of standard or certified reference materials. For compositional analysis of crops, in the case of an analyte for which no previous method exists, it is extremely difficult, if not impossible, to obtain any kind of reference or certified material, nor are there proficiency programs available. In some cases standards are able to be custom synthesized with additional

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expense. This was the case for the two cyclopropenoic fatty acids, malvalic and sterculic, that were synthesized by Gateway Chemical Technology for Monsanto Co. for the development of a quantitative method for cyclopropenoid fatty acids in cottonseed.[10] Additionally, because the compounds of interest are endogenous to the matrix, the level of analyte often varies, considerably in some cases, depending on the geography, climate, and soil where the crop was grown.

Developing and optimizing methods for endogenous compounds usually requires a great deal of experimentation for the extraction and isolation phase of the method. There may be several methods available to identify and quantify specific compounds, especially for standards or "clean" matrices, but ensuring that all of the analytes of interest have been extracted from the matrix is critical for the analysis of complex matrices, such as crops.

The costs involved in developing and validating methods are high. For the contract laboratory, these are not feasible without some assurance that there will be a sufficient volume of analyses to perform on a routine basis. Contract laboratories typically analyze many different types of food samples, from crops to finished foods to raw materials, some of which have a wide variety of ingredients, or components, with various compositional structures and varying levels of analyte. As validation of each particular matrix is neither cost-effective nor feasible, validations are often performed on groups of matrices that have similar properties or compositions.

Biotechnology companies invest substantial dollars in developing genetically engineered crops. Study data for compositional analysis is significant in that it shows that the genetically engineered crop is compositionally "equivalent" to conventional crops. Equivalence is often understood to mean that compositional data will be consistent, per crop, per analyte. This phrase can pose certain problems, depending on how strictly one interprets the word consistent. The most significant of these, as previously stated, is that crops inherently have variable composition or nutrient levels, depending on the soil, climate, and location. Depending on the analyte level, method, and instrument used, there will also be analytical variation, both within and between laboratories, resulting in more variation of the data. There are several sources available that provide the nutrient content for various crops. Some of these are the USDA Plants Database,[11] the ILSI Crop Composition Database,[12] and the FAO Nutrient Response Database.[13]

Second, analytical methodologies are constantly evolving to include new methods, techniques, and instruments that are more accurate, cost-efficient, safer, and environmentally friendly. Although historical data are used as a basis for comparing compositional data, there must be a forum and process for making the change to newer methodology, when that methodology has been proven to be more accurate. This raises the question of whether uniform methodology should be implemented for data that are contributed to public databases such as the ILSI Crop Composition Database (www.cropcomposition.org)[12] and the methods chosen on the basis of the collaborative work of invested parties.

CONCLUSION

Method development and validation are both very time-consuming endeavors, and documentation is critical for both phases. For the developmental phase, documentation is important to create an organized, systematic approach for experimentation, as well as to maintain a historical narrative of the development process. Once a method has been developed, a formal method and validation protocol are written. When these are approved by management, the method validation can begin. After the validation starts, there can be no changes to (deviations from) the method or protocol without justification and written approval. All validation results and supporting documentation including observations and deviations are included in a validation report. If updates or changes to the method are needed, as a result of the validation, these are done at this time. After the validation report and method updates have been made and approved, the method is then locked, personnel can be trained, and the method is placed into service.

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Notes

The authors declare no competing financial interest.

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