AOAC SMPR® 2022.003

AOAC *Standard Method Performance Requirements* (SMPRs®) for Determination of Biological Spices and Botanicals, and Relevant (Common) Biological Adulterants

Intended Use: Surveillance and Monitoring by Trained Analysts

1 Purpose

AOAC SMPRs are consensus standards developed in accordance with AOAC policy, AOAC Due Process for Development of AOAC Non-Method Consensus Standards and Documents. SMPRs describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation (SLV), a multisite collaborative study, or another AOAC-approved study design for method characterization and validation. SMPRs are written and adopted by AOAC through its stakeholder-based integrated science programs and projects, which are composed of representatives and experts from the academic, government, industry, and nonprofit sectors. AOAC SMPRs may be used to develop validation studies along with validation guidance to validate and optimized methods. They are also used by AOAC method review experts, including expert review panels (ERPs), in their evaluation of validation study data for methods being considered for AOAC *Performance Tested Methods*SM, *Reviewed and Recognized*SM, or AOAC *Official Methods of Analysis*SM, and can be used as acceptance criteria for verification at user laboratories.

2 Applicability

This SMPR contains assessment parameters on the performance of molecular applications to monitor spices and botanicals for the probable presence of economically motivated biological adulterants (EMBA).

This SMPR is designed to evaluate next-generation sequencing methods (NGS) developed to assess potential economic adulteration in defined commodities. The SMPR is purposely designed with general descriptions to be applicable to a broad range of innovative sequencing platforms and concepts. The identified species on defined spice or botanical samples will be used to perform the evaluations of the method's performances by the ERP.

The analytical results gather all the parts/tissues of a plant that share the same DNA. Therefore, specific parts of plants used for botanicals and spices (e.g., bark, bud, stigma, seed, fruit, leaf) cannot be differentiated based on DNA sequences. By definition a spice/botanical is a single specific part of a plant; EMBA may be both endogenous or exogenous materials.

Note: The endogenous material corresponds to the floral/plant waste belonging to the plant to which the spice/botanical belongs. Regarding economically motivated adulteration, endogenous adulterants can be raw plant (e.g., saffron's stamen/petal, sticks, stems) or processed plant material (e.g., exhausted/spent spice). The exogenous material corresponds to all materials that are not part of the plant to which the spice originates.

In that respect, only exogenous adulterants can be detected using DNA methods. The certificates of analysis should not mention any spice or botanical name, but only the Latin binomial name of the detected plant. When a detected adulterant cannot be identified at the species taxonomic level, the result can be displayed using other taxonomic levels (e.g., family, genus).

Complete documentation of the authentic samples used to build the database, target genes, primers, and DNA data analysis are to be supplied by the method authors. The scope of the method is defined by the applicable database of the NGS method, matrixes, and concentration range of applicable operation (e.g., spices, botanicals); expansion of the scope is possible with the inclusion of additional authentic samples into the database and validation using the performance characteristics described in this SMPR.

3 Analytical Technique

The identification method is based on DNA sequencing to evaluate spices and botanicals for possible EMBAs. Any NGS method, with appropriate database and data analysis concept, that will identify the species content of defined samples is considered. The analysis report should provide the list of identified taxonomic species of the analyzed samples. The method shall demonstrate reliability using the requirements listed in this SMPR.

The performance characteristics of the DNA sequencing method are defined by the content of the database, defined primers, selectivity (specificity), ability to distinguish the taxonomic species in a mix of species, and reliability of the identification results.

4 Definitions

Applicability statement.—General statement about the intended purpose and scope of the method, entailing key aspects of expected achievements for the specific situation and circumstances. Key points to cover are the intended scope, purpose, and indication of probability of identification.

Authentic samples.—Samples representative of the genuine commodity. These samples should represent the spices or botanicals variability seen naturally in the commodity. The authentic samples will be used to properly define the method testing scope.

Botanical and spices.—Refer to plants or botany. May refer to the whole plant, part of the plant (e.g., bark, woods, leaves, stems, roots, rhizomes, flowers, fruits, seeds, etc.), or extract of the parts.

EMBA.—Fraudulent addition of nonauthentic (nondeclared) substances or removal or replacement of authentic (declared) substances without the purchaser's knowledge for economic gain of the seller.

Exclusivity.–Ability of an identification method to correctly reject nontarget materials.

Identification method.—Any qualitative method that reliably analyzes a botanical or species material and returns a taxonomical identification of the components.

Identification (taxonomic identification).—Taxonomic assessment of the species content of the product being analyzed.

In silico analysis.—The use of computer simulation to evaluate target and non-target sequences for molecular methods.

Inclusivity.—Ability of an identification method to correctly identify variants species of the target group(s) that meet the identity specification.

Multilaboratory validation (MLV).—Demonstration among laboratories using adulterated samples created by a third-party group and supplied blindly to the participating laboratories.

NGS.—Analytical technology using specific DNA sequencers to obtain sequencing data. The data are usually composed by multiple

sequences obtained by parallel sequencing, and the output is a file containing all sequences.

SLV.—Demonstration by one laboratory of method performance on the validation samples.

5 Method Performance Requirements

5.1. In silico analysis.—Performance requirements are described for the *in silico* analysis (Table1).

The report should assess all the performance characteristics as a whole, and a final conclusion should be provided together with the limitations.

5.2. Requirements for matrix study.—As already mentioned, qualitative analytical results of identified species on defined samples are only taken into considerations. The proposed approach is taking into account pragmatic considerations to find the right balance between the costs of the study and appropriate performances assessment of the qualitative NGS method. The matrix study enables assessment of the performances of the sample prep and analytical workflow; this part can only be conducted after a successful *in silico* analysis. Various mixes of adulterants and authentic samples should be tested for a given species of authentic samples.

For a single species of spices or botanicals claim, five variants of the authentic samples shall be run to ensure the required quality controls and make sure the method is able to correctly identify these five variants. No other species shall be identified in the authentic samples as these materials will be used to prepare the mixes of authentic sample and adulterant. In addition, multiple different relevant mixes of plant adulterant and authentic samples shall be tested. Consider an appropriate number of relevant plant adulterants per tested authentic sample; usually, no more than 10 possible adulterants are expected. The mixes should be done in most of the cases with at least 10% adulterant and 90% authentic samples. However, if relevant and realistic, it is possible to decrease the ratio of adulterant in a tested mix, e.g., 5 % adulterant and 95% authentic sample; a modification of this ratio should be motivated with a proper rationale. Whenever possible, use adulterants from different geographical regions (origin) to produce the mixes replicates. Whenever possible and relevant, prioritize assessing closely related taxa that could be used as adulterants. The procedure to produce the mixes shall be documented and reproducible. A minimum of 25 test results is recommended to be generated with equally distributed replicates among the various mixes. Together with pure authentic samples, a total of 30 test results shall be produced.

It is recommended to prioritize the authentic samples and related plant adulterants defined within the AOAC SMPRs® on Nontargeted Testing (NTT).

Illustrations of the study design are given in Table 2 using *Curcuma longa*, i.e., turmeric, as authentic sample together with its relevant plant adulterants; Table 3 using *Crocus sativus*, i.e., saffron, as authentic sample together with its relevant plant adulterants.

However, it is expected to cover broader claims, fitting with the database content and the *in silico* analysis outcomes. The possible claims and the required testing are presented Table 4. Again, the replicates shall be equally distributed using relevant mixes of authentic samples and plant adulterants.

No failure of identifying an adulterant is expected for a claim restricted to one species and related adulterants. Any outlying data should be explained with proper root cause analysis. For instance, repeat the testing to discard any possible sample preparation or operator error and/or run the appropriate in silico analysis linked to this outlier event.

No more than 5% failure is expected to support claims that are not limited to one single authentic sample and related adulterants; i.e., *(1)* selected species and their related adulterants, *(2)* variety of species and their related adulterants, and *(3)* broad range of species and their related adulterants. The observed unexpected results should be distributed among various species and should be explained with the support of the *in silico* analysis (*see* section 5.1).

6 System Suitability Tests and/or Analytical Quality Control

Suitable methods will include blanks and appropriate check standards.

7 Method Validation Material(s) and Required Information Prior Starting the Study

Scope of the method.

For the *in silico* analysis (section 5.1): Target DNA region(s); primer selection and design; database content; algorithm concept; limitations.

For the matrix study (section 5.2): Protocols used to identify reference materials as authentic and to create adulterated samples; study design with the list of tested authentic samples, the mixes with plant adulterants with the number of replicates.

8 Validation Guidance

Official Methods of Analysis of AOAC INTERNATIONAL, "Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis." Available at http://www.eoma.aoac.org/app_d.pdf

Official Methods of Analysis of AOAC INTERNATIONAL, "Appendix K: AOAC Guidelines for Validation of Botanical Identification Methods." Available at http://www.eoma.aoac.org/app_k.pdf

Official Methods of Analysis of AOAC INTERNATIONAL, "Appendix Q: Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern *In Silico* Approaches." Available at http://www.eoma.aoac.org/app_q.pdf

ISO/CD 22949-1.3:2020—Molecular biomarker analysis—Methods of analysis for the detection and identification of animal species in foods and food products (nucleotide sequencing-based methods)—Part 1: General requirements

Additional guidance:

For SLV studies, the method should be evaluated using an *in silico* analysis and testing on prescribed authentic and adulterated materials. Methods approved at this level may proceed to a second level of evaluation: blinded samples containing unknown adulterants should be sent to laboratories participating in an MLV study/proficiency testing/or innovative approach that could be proposed.

9 Maximum Time-to-Result

No maximum time.

Acknowledgment of the Draft Group

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Table 1. Performance requirements for *in silico* analysis

Target DNA region(s)	Target DNA region(s) should be specific of taxonomic species included in database. Region(s) and length(s) of target DNA region should be selected to avoid nonamplification event.
Primer selection and design	Quality of selected primers should be assessed regardless of universality, secondary structures, unimolecular folding, partial match and mismatch, hairpins, GC content, or number of degenerations. *Note*: Limitations should be highlighted to end-users.
Database content	DNA database content defines scope of identification method. Sequences available in database shall come from authentic samples, and origin of entries should be available. It is advised to obtain several representatives (entries) per species to the extent possible. Therefore, database should provide the following information: database version, list of genera and species, number of different entries for each species, origin of entries, description of types of DNA sequences, e.g., one unique sequence issued from average of various sequences of the same species or several sequences of various entries, list of closely related species and/or variants that are not differentiated by identification method; list of species which target region has <100% DNA homology with the selected primers.[a] Database content should be available to end users.
Algorithm	Algorithm should be described, and version should be provided.
Evaluation of nontarget DNA sequences	DNA sequences from nontarget species (plants or possible other adulterants) that could be used for end-product should be assessed. Two types on nontarget DNA sequences should be evaluated: *(1)* close species and relevant and *(2)* excipients. Minimum number of mismatches should be defined as acceptable for exclusivity.[b]
Limitations	Highlight any possible restrictions, e.g., possible treatments of spices and botanicals that might impact analysis and quality of botanicals, spices or botanicals format that might be challenging to analyze, lack of appropriate entries for some species, etc. Information about limitations shall be included in submission and made available to end-users.

[a] In case of doubt regarding efficiency of amplification (amplificability) for these species, inclusivity testing with relevant variants might be required.

[b] In case of doubt for some nontarget species, exclusivity with relevant variants testing might be required.

Table 2. Study design for single species of spices or botanicals claim with five relevant plant adulterants[a]

Authentic samples, i.e., *Curcuma longa* (turmeric), %	Adulterants	Tests (equally distributed among adulterants as much as possible)[b]	Test results
100	0%	5 Replicates as quality controls	5
90	10% *Curcuma xanthorrhoea*	N_1 (e.g., 5 replicates)	25 Mixes of authentic samples and adulterants
90	10% *Curcuma zedoaria*	N_2 (e.g., 5 replicates)	
90	10% *Curcuma malabarica*	N_4 (e.g., 5 replicates)	
90	10% *Curcuma aromatica*	N_5 (e.g., 5 replicates)	
90	10% Cassava (*Manihot esculenta*)	N_6 (e.g., 4 replicates)	
Total data sets			30

[a] Table from AOAC SMPR 2021.011 Nontargeted Testing (NTT) of Ingredients for Food Authenticity/Fraud Evaluation of Turmeric.

[b] N_x = Number of replicates.

Table 3. Study design for single species of spices or botanicals claim with seven relevant plant adulterants[a]

Authentic samples, i.e., *Crocus sativus* (saffron), %	Adulterants	Tests (equally distributed among adulterants as much as possible)[b]	Test results
100	0%	5 Replicates as quality controls	5
90	10% Safflower stigmas	N_1 (e.g., 3 replicates)	25 Mixes of authentic samples and adulterants
90	10% Marigold stigmas	N_2 (e.g., 3 replicates)	
90	10% Dyed corn stigmas	N_3 (e.g,. 3 replicates)	
90	10% Sandalwood	N_4 (e.g., 4 replicates)	
90	10% Campeche wood powder	N_5 (e.g., 4 replicates)	
90	10% Gardenia fruit	N_6 (e.g., 4 replicates)	
90	10% Curcuma	N_7 (e.g., 4 replicates)	
Total data sets			30

[a] Table from AOAC SMPR 2021.012 Nontargeted Testing (NTT) of Ingredients for Food Authenticity/Fraud Evaluation of Saffron.

[b] N_x = Number of replicates.

Table 4. Possible scopes of method and required testing

Scope of method	Number of spices or botanicals claim	Replicates of quality controls (authentic samples)	Replicates of mixes of authentic samples and adulterants	Total data set
One species and related adulterants	1	5 Variants	25	30
Selected species and their related adulterants	≥5	≥5 × 5 Variants	≥5 × 25	≥150
Variety of species and their related adulterants	≥10	≥10 × 5 Variants	≥10 × 25	≥300
Broad range of species and their related adulterants	≥20	≥20 × 5 Variants	≥20 × 25	≥600