AOAC SMPR 2021.XXX

Method Name: **Determination of biological spices and botanicals, and relevant (common) biological adulterants**

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Intended Use:

AOAC *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, or a multi-site collaborative study.

SMPRs are written and adopted by AOAC using the consensus of stakeholder panel composed of representatives from industry, regulatory organizations, academic and/or research institutions, service laboratories and method developers. AOAC SMPRs are used by AOAC expert review panels (ERPs) in their evaluation of validation study data for method being considered for AOAC *Performance Tested Methods*^{SM} or AOAC *Official Methods of Analysis*^{SM} and can be used as acceptance criteria for verification at user laboratories.

1. 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 Expert Review Panel.

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; economically motivated biological adulterants may be both endogenous or exogenous materials.

Note: The endogenous material corresponds to the floral/plant waste belonging to the plant which spice/botanical belongs to. 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, the 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, the 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.

2. 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.

For single lab validation 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 a multi-laboratory validation study/proficiency testing/or innovative approach that could be proposed.

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

3. Definitions:

Applicability statement – A 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, the purpose, and an 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, a part of the plant (e.g., bark, woods, leaves, stems, roots, rhizomes, flowers, fruits, seeds, etc.), or an extract of the parts.

Economically motivated biological adulteration (EMBA) – The fraudulent addition of non-authentic (non-declared) 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 non-target materials.

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

Identification method – An identification method is any qualitative method that reliably analyzes a botanical or species material and returns a taxonomical identification of the components.

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

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

Multi-laboratory validation – Demonstration between laboratories using adulterated samples created by a third-party group and supplied blindly to the participating laboratories.

Next generation sequencing (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.

Single laboratory validation – Demonstration by one laboratory of method performance on the validation samples.

4. Method Performance Requirements:

4.1. *In silico* analysis

The performance requirements are described for the *in-silico* analysis (Table1).

Table 1: Performance requirements for *in silico* analysis

Target DNA region(s)	The target DNA region(s) should be specific of the taxonomic species included in the database. The region(s) and length(s) of the target DNA region should be selected to avoid non-amplification event.
Primer selection and design	The quality of the selected primers should be assessed regardless their universality, secondary structures, unimolecular folding, partial match and mismatch, hairpins, GC content, number of degenerations. Note: limitations should be highlighted to the end-users
Database content	The DNA database content defines the scope of the identification method. The sequences available in the database shall come from authentic samples, and the origin of the entries should be available. It is advised to get several representatives (entries) per species to the extent possible.

	Therefore, the database should provide the following information: — the database version, — the list of genera and species, — the number of different entries for each species, — the origin of the entries, — the description of the types of DNA sequences, e.g. one unique sequence issued from the average of various sequences of the same species or several sequences of various entries, — the list of the closely related species and/or variants that are not differentiated by the identification method; — the list of species which target region has less than 100% DNA homology with the selected primers[a]. The database content should be available to the end-users.
Algorithm	The algorithm should be described, and the version should be provided.
Evaluation of non-target DNA sequences	DNA sequences from non-target species (plants or possible other adulterants) that could be used for the end-product should be assessed. Two types on non-target DNA sequences should be evaluated: (i) close species and relevant, (ii) excipients A minimum number of mismatches should be defined as acceptable for the exclusivity[b].
Limitations	Highlight any possible restrictions, e.g. possible treatments of spices and botanicals that might impact the analysis and quality of the botanicals, spices or botanicals format that might be challenging to analyze, lack of appropriate entries for some species, etc... The information about the limitations shall be included in the submission and made available to the end-users.

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

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

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

4.2. 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, 5 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 5 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% of 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 Non-Targeted Testing (NTT).

Illustrations of the study design are given in

- Table 2a using *Curcuma longa*, i.e. turmeric, as authentic sample together with its relevant plant adulterants;
- Table 2b using *Crocus sativus*, i.e. saffron, as authentic sample together with its relevant plant adulterants.

Table 2a: Study design for a single species of spices or botanicals claim with 5 relevant plant adulterants *(Table from the AOAC SMPR 2021.XXX; Draft AOAC Standard Method Performance Requirements (SMPRs) for Non-Targeted Testing (NTT) of Ingredients for Food Authenticity/Fraud Evaluation of Turmeric*

Authentic samples, i.e. *Curcuma longa* (Turmeric)	**Adulterants**	**Tests (equally distributed among the adulterants as much as possible)**	**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**

With N_x corresponding to number of replicates

Table 2b: Study design for a single species of spices or botanicals claim with 7 relevant plant adulterants *(Table from the AOAC SMPR 2021.XXX; Draft AOAC Standard Method Performance Requirements (SMPRs) for Non-Targeted Testing (NTT) of Ingredients for Food Authenticity/Fraud Evaluation of Saffron*

Authentic samples, i.e. *Crocus sativus* (Saffron)	**Adulterants**	**Tests (equally distributed among the adulterants as much as possible)**	**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**

With N_x corresponding to number of replicates

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 3. Again, the replicates shall be equally distributed using relevant mixes of authentic samples and plant adulterants.

Table 3: Possible scopes of the method and required testing

Scope of the 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 replicates	30
Selected species and their related adulterants	≥ 5	≥ 5 x 5 variants	≥ 5 x 25 replicates	≥ 150
Variety of species and their related adulterants	≥ 10	≥ 10 x 5 variants	≥ 10 x 25 replicates	≥ 300
Broad range of species and their related adulterants	≥ 20	≥ 20 x 5 variants	≥ 20 x 25 replicates	≥ 600

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. (i) selected species and their related adulterants, (ii) variety of species and their related adulterants, (iii) 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 sub-clause 4.1).

5. System suitability tests and/or analytical quality control:

Suitable methods will include blanks, and appropriate check standards.

6. Method validation material(s) and required information prior starting the study:

Scope of the method.

For the *in-silico* analysis (sub-clause 4.1): Target DNA region(s); Primer selection and design; Database content; Algorithm concept; Limitations.

For the matrix study (sub-clause 4.2): 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.

7. Validation Guidance:

AOAC INTERNATIONAL Appendix D: Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis, version 2002

AOAC INTERNATIONAL Appendix K: AOAC Guidelines for Validation of Botanical Identification Methods, version 2013

AOAC INTERNATIONAL Appendix Q: Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern In- Silico Approaches, version 2020

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

8. Maximum Time-To-Result:

No maximum time**.**