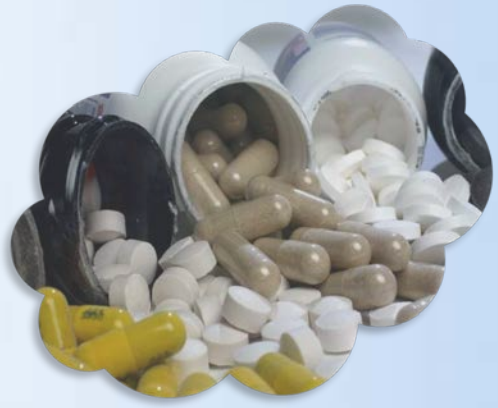
DECEMBER 9-10, 2015
AOAC STAKEHOLDER PANEL on DIETARY SUPPLEMENTS (SPDS)

EXPERT REVIEW PANEL (SET 2) MEETING BOOK



AOAC INTERNATIONAL H
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Expert Review Panels

The ERPs review and approve appropriate methods (as submitted or modified) for adoption as First Action Official Methods or for further validation. ERPs also make recommendations regarding Final Action Official Methods status.

Expert Review Panels

- Must be supported by relevant stakeholders.
- Constituted for the review of methods, not for Standard Method Performance Requirements (SMPR) purposes or as an extension of a Working Group.
- Consist of a minimum of seven (7) members representing a balance of expert stakeholders. **Quorum is a minimum of 7 members present or 2/3 of the total vetted members, whichever is greater.**
- ERP constituency must be approved by the Official Methods Board (OMB).
- Holds transparent public meetings only.
- Remains in force as long as method in First Action Status.

First Action Official Method Status decision

- Must be made by an ERP constituted or reinstated post 2011-03-28 for First Action Official Method Approval (FAOMA).
- Must be made by an ERP vetted for FAOMA purposes by OMB post 2011-03-28.
- Method adopted by ERP must perform adequately against the SMPR set forth by the stakeholders. Or demonstrate performance or characteristics that meet the scope, applicability and/or claims of the method.
- Method must be adopted by unanimous decision of ERP on first ballot, If not unanimous, negative votes must delineate scientific reasons.
- Negative voter(s) can be overridden by 2/3 of non-negative voting ERP members after due consideration
- Method becomes First Action Official Methods on date when ERP decision is made.
- Methods to be drafted into AOAC format by a knowledgeable AOAC staff member or designee in collaboration with the ERP and method author.
- Report of FAOMS decision complete with ERP report regarding decision including scientific background (references etc) to be published concurrently with method in traditional AOAC publication venues.

Method in First Action Status and Transitioning to Final Action Status

- Further data indicative of adequate method reproducibility (between laboratory) performance to be collected. Data may be collected via a collaborative study or by proficiency or other testing data of similar magnitude.
- Two years maximum transition time (additional year(s) if ERP determines a relevant collaborative study or proficiency or other data collection is in progress).
- Method removed from First Action Official Methods and OMA if no evidence of method use available at the end of the transition time.
- Method removed from First Action Official Methods and OMA if no data indicative of adequate method reproducibility is forthcoming as outlined above at the end of the transition time.
- ERP to recommend Method to Official Final Action Status to the OMB.
- OMB decision on First to Final Action Status

Revised October 2013

Online Technical Resources

Method Development, Optimization & Validation

- OMA - Appendix F - Guidelines for Standard Method Performance Requirements
- Homogeneity
- Guide for Writing Methods in AOAC Format
- Statistics Protocol Review Form
- OMA - Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis
- OMA - Appendix G: Procedures and Guidelines for the Use of AOAC Voluntary Consensus Standards to Evaluate Characteristics of a Method of Analysis
- OMA - Appendix I: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Biological Threat Agent
- Methods and/or Procedures
- OMA - Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces
- OMA - Appendix K: Guidelines for Dietary Supplements and Botanicals
- OMA - Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation
- OMA - Appendix M - Validation Procedures for Quantitative Food Allergen ELISA Methods: Community Guidance and Best Practices
- Safety Checklist

Method Review

- Examples of Statistical Analysis
- Statistics Manuscript Review Form
- OMA - Appendix A: Standard Solutions and Reference Materials
- OMA - Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis
- OMA - Appendix H: Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods

Miscellaneous

- Definition of Terms and Explanatory Notes
- OMA - Appendix B: Laboratory Safety
- OMA - Appendix E: Laboratory Quality Assurance
- OMA - Appendix C: Reference Tables

All resources are accessible at
http://www.aoac.org/vmeth/guidelines.htm

For questions, please contact:
P 301-924-7077 x157 E dmckenzie@aoac.org

About Expert Review Panels (ERPs)

ERP OVERVIEW:

An Expert Review Panel (ERP) is assembled to review and adopt methods as Official First Action. ERPs will track Official Methods for two years or until such time as reproducibility has been demonstrated and cumulative feedback on method use and performance are obtained. ERPs will make a recommendation regarding Final Action method status for all OMAs to the Official Methods Board (OMB).

All ERP members are expected to serve with the highest integrity and without direct or indirect conflicts of interest. A method assignment can last two years. All members of the ERP are expected to actively participate in ERP meetings and to perform duties and reviews in timely fashion. All members should maintain strict adherence to review timelines and deadlines. AOAC staff documents ERP deliberations.

ESTABLISHING AN EXPERT REVIEW PANEL:

- AOAC staff issues a Call for Experts:
  - Based on voluntary consensus standards and methods submitted to AOAC INTERNATIONAL that may meet the standards.
  - Proprietary and sole source method developers submit individual methods to the AOAC Research Institute.
  - Candidates are asked to submit a CV or information that demonstrates expertise to AOAC staff if not already part of a recognized pool of experts.
- AOAC Chief Scientific Officer (CSO) reviews the documentation for the candidates and make recommends a slate for an expert review panel including the chair to the Official Methods Board.
- The candidate list and supporting documentation are forwarded to the Chair of the OMB who will assign the review to at least two OMB members.
- The OMB reviewers will review the candidates for expertise and perceived conflicts of interest and the OMB may then approve the members of the ERP. A Chair for the ERP is also approved.

EXPERT REVIEW PANEL (ERP):

- Review, discuss and demonstrate consensus on methods for Official First Action method status.
- Participate in the publications process of First Action methods.
- Track and discuss feedback all First Action methods for two years.
- Reach and demonstrate consensus on recommendations for Final Action method status.
- Actively participate in the broader stakeholder effort.

ERP CHAIR:

- Lead ERP discussions in the review and adoption of methods for First Action Official Methods.
- Participate in stakeholder panel activities.
- Review and approve ERP report.
- Work with AOAC staff, working groups and other stakeholder panels to ensure a thorough understanding of the standard method performance requirements and the methods to be assessed.
- Implement the OMB First Action to Final Action Guidelines with the ERP members.
- Advise and review First Action methods and post First Action publications.
- Represent the ERP in presenting the ERPs recommendation to the Official Methods Board regarding Final Action method status.

MECHANICS OF AN AOAC EXPERT REVIEW PANEL

- AOAC CSO assigns methods for review to the expert review panel members.
- For each method, 2 ERP members are assigned as primary and secondary reviewers and present at the ERP meeting.
- All members are expected to actively participate and review methods for First Action Official Method status - conducting thorough and prompt review of methods and being prepared to speak on assigned methods at ERP meetings
- The ERP chair and the 2 reviewers for each method are expected to participate in the publications peer review process for First Action methods.
- ERP reviewers track assigned methods that were adopted as First Action Official Methods and update ERP on method use during two year period between First Action and Final Action
  ERP members are expected to participant in the stakeholder panel activities and/or community at large .

- *ERPs can work with topic advisors (aka, subject matter experts)*
- *OMB can recognize a pool of experts from which ERP members can be selected*

Eligibility Criteria for Expert Reviewers

Be a key expert and/or thought leader of the method or priority under consideration.

- Demonstrated knowledge in the appropriate scientific disciplines.
- Demonstrated knowledge regarding data relevant to adequate method performance.
- Demonstrated knowledge of practical application of analytical methods to bona fide diagnostic requirements.

Be approved by the Official Methods Board

- Qualifications must be clearly described and submitted to AOAC headquarters.

Duties of Expert Reviewers

Members of the Pool of Experts will be called upon to serve on ERPs as needed and to review documents .These documents may include:

- Procedural documents on how methods will be selected and how single laboratory validation studies will be done;
- Methods submitted for consideration as First Action Official Methods;
- Methods submitted for selection for further validation studies;
- Protocols to be used for single laboratory validation studies;
- Selection of methods to be considered for full collaborative studies; and
- Validation study reports

Revised October 2013

AOAC INTERNATIONAL

AOAC Expert Review Panel for AOAC Stakeholder Panel on Dietary Supplements (SPDS) Set 2 Ingredients: Ashwagandha, Folin C, and Kratom

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LIST OF METHODS:

- ASH-01: Estimation of Withanolides (Withanoside IV, Withanoside V, Withaferin A, 12-Deoxywithastromonolide, Withanolide A, Withanolide B) in Withania somnifera
    - Submitted by Balasuramanian Murali, Natural Remedies, India

- FOL-01: Single Reagent Folin
    - Submitted by Joe Vinson, University of Scranton, USA

- FOL-02: METHOD FOR THE ESTIMATION OF TOTAL PHENOLIC CONTENT USING THE FOLIN- C ASSAY
    - Submitted by Jyotish Srivastava, OmniActive Health Technologies, India

- FOL-03: Modified Folin-C Antioxidant Capacity Assay for Measuring Lipophilic Antioxidants
    - Submitted by Resat Apak, Istanbul University, Turkey

- KRA-01: Quantitative and Qualitative Analysis of Mitragynine in 'Kratom" (Mitragyna Speciosa) by GC-MS, LC-MS/MS and UPLC-PDA
    - Submitted by Christine Casey, US FDA

- KRA-02: Quantification of Mitragynine in Kratom Raw Materials and Finished Products by High-Performance Liquid Chromatography: Single-Laboratory Validation
    - Submitted by Elizabeth Mudge, British Columbia Institute of Technology, Canada

- KRA-03: Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of *Mitragyna speciosa* Korth Using Liquid Chromatography
    - Submitted by Iklas Khan, University of Mississippi, USA

- KRA-04: LC/MS Method for the Identification of Mitragyna speciose (Kratom) and Quantitiation of Mitragynine Using Linear Ion Trap Mass Spectrometer
    - Submitted by Teresa Cain, US FDA

Set 2ERP Rosters

ASHWAGANDHA		FOLIN-C		KRATOM	
Anton Bzhelyansky	USP	Nour Eddine Es-Safi	Mohammed V University in Rabat	Christine Casey	FDA
Nour Eddine ES-SAFI	Mohammed V University in Rabat	John Finley	Louisiana State University	Nour Eddine ES-SAFI	Mohammed V University in Rabat
Prashant Ingle	Herbalife	Prashant Ingle	Herbalife	Charles Metcalfe	Custom Analytics
Tom Phillips	State of Maryland	Martha Jennens	Covance	Tom Phillips	State of Maryland
Catherine Rimmer	NIST	Dana Krueger	Krueger Food Laboratories	Catherine Rimmer	NIST
Casey Sayre	Roseman University of Health Sciences	Jungmin Lee	USDA	Darryl Sullivan	Covance
Aniko Solyom	GAAS Analytical	Tom Phillips	State of Maryland	John Szpykla	Merieux Nutrisciences
Darryl Sullivan	Covance	Catherine Rimmer	NIST	Yanhong Wang	University of Mississippi
Kurt Young	GNC/Nutra Manufacturing	Aniko Solyom	GAAS Analytical		
Yanjun Zhang	Herbalife	Darryl Sullivan	Covance		
		Joseph Zhou	Sunshineville Health		

The Scientific Association Dedicated to Analytical Excellence®

AOAC Stakeholder Panel on Dietary Supplements

SET 2 INGREDIENTS EXPERT REVIEW PANEL
(Ashwagandha, Folin C, Kratom)

Wednesday, December 9, 2015

A G E N D A

EXPERT REVIEW PANEL CHAIR: Darryl Sullivan, Covance

1. Welcome and Introductions (1 :00 p.m. – 1 :10 p.m.)
   Darryl Sullivan, Covance (ERP Chair)

2. Review
   A. AOAC Volunteer Policies & ERP Proccess Overview and Guidelines (1 :10 p.m. – 1 :30 p.m.)
      Deborah McKenzie

3. Review of Methods

   For each method the assigned ERP members will present a review of the revised method manuscripts, after which the ERP will discuss the method and render a decision on the status for each method.

   A. Kratom (December 9, 1:30 p.m. – 5 :00 p.m.)
      a. KRA-01
      b. KRA-02
      c. KRA-03
      d. KRA-04
      e. Final Action Requirements for Approved Method(s)

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AOAC Stakeholder Panel on Dietary Supplements

SET 2 INGREDIENTS EXPERT REVIEW PANEL (Ashwagandha, Folin C, Kratom)

Thursday, December 10, 2015

A G E N D A

EXPERT REVIEW PANEL CHAIR: Darryl Sullivan, Covance

1. Welcome and Introductions (9 :00 a.m. – 9 :10 a.m.)
   Darryl Sullivan, Covance (ERP Chair)

2. Review
   A. AOAC Volunteer Policies & ERP Proccess Overview and Guidelines (9 :10 a.m. – 9 :30 a.m.)
      Deborah McKenzie

3. Review of Methods

   For each method the assigned ERP members will present a review of the revised method manuscripts, after which the ERP will discuss the method and render a decision on the status for each method.

   A. Ashwagandha (December 10, 9:30 a.m. – 10 :30 a.m.)
      a. ASH-01
      b. Final Action Requiremets for Approved Method(s)

   B. Folin-C (December 10, 10 :30 a.m. – 2 :00 p.m.)
      a. FOL-01
      b. FOL-02
      c. FOL-03

4. Adjourn (2 :00 p.m.)

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MEMORANDUM

DATE: **DECEMBER 9, 2015**

TO: **SPDS SET 2 EXPERT REVIEW PANEL MEMBERS (KRATOM)**

FROM: **AOAC INTERNATIONAL**

SUBJECT: **KRATOM METHOD SUBMISSIONS AND REVIEWS**

BACKGROUND:

Four methods were submitted in response to the Kratom Call for Methods. The reviews submitted are provided in this meeting book, and links to the *Standard Method Performance Requirements*℠ (SMPRs) and the candidate methods themselves are provided below.

AOAC Candidate Method Number	Submitter	Primary Reviewer	Secondary Reviewer(s)
KRA-01	US FDA	Yan-Hong Wang	Tom Phillips
KRA-02	BCIT	Nour Eddine Es-Safi	Charles Metcalfe
KRA-03	University of Mississippi	Tom Phillips	Christine Casey; John Szpylka
KRA-04	US FDA	Yan-Hong Wang	Kate Rimmer

SMPRs:

- AOAC SMPR 2015.008 – *Standard Method Performance Requirements*℠ for Alkaloids of *Mitragyna speciosa.*

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Yon-Hong Wang

Title of Method: Quantitative and Qualitative Analysis of Mitragynine in "Kratom" (Mitragyna Speciosa) by GC-MS, LC-MS/MS and UPLC-PDA

AOAC Candidate Method Number: KRA-01

Applicable SMPR : AOAC SMPR 2018.008

I. SUMMARY OF METHOD

GC/MS, UPLC-PDA and LC-MS/MS methods were developed for qualitative and quantitative analysis of mitragynine in Mitragyna speciosa (Kratom) and realted products. UPLC-PDA and LC-MS/MS methods were validated by characterizing a dry leaf Kratom product. The developed methods were applied for the analysis of small packets of drinks, capsules, tea leaves, powdered leaves and spent leaves from a manufacturing processing facility.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   Yes.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   Yes.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   LOD and LOQ are not specified in the method.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   The method, as written, does not contain all appropriate precautionary and warning.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   LOD and LOQ are not determined in the method.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   The method does not demonstrate information of parameters including bias, recovery, and LOQ.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   Reference Material hasn't been specified in the method.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   The method was validated, but some parameters including recovery, LOD and LOQ were not specified.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

The method should be further validated for the recovery.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   Yes.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   Yes.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes.

5. *Based on the supporting information, what are pros/strengths of the method?*

   The developed GC and LC methods are suitable for the analysis of mitragynine in Mitragyna speciosa and related products. The LC method is partial validated.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   The LC method is partial validated. Validation of parameters including recovery, LOD and LOQ should be included.

7. *Any general comments about the method?*

   The developed GC and LC methods are suitable for the analysis of mitragynine in Mitragyna speciosa and related products.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   This method should be further validated.

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Tom Phillips

Title of Method: Quantitative and Qualitative Analysis of Mitragynine in "Kratom" (Mytragyna speciosa) by GC-MS, LC-MS/MS and UPLC-PDA

Aoac Candidate Method Number: KRA-01

Applicable SMPR : 2015.008

I. SUMMARY OF METHOD

The leaves of Mitragyna speciosa consist of two primary active alkaloids: Mitragynine 66.2%, and 7 α -hydroxy-7H-mitragynine 2.0%, and three indole alkaloids: Paynantheine 8.6%, Speciogynine 6.6%, and Speciociliatine 0.8%. Since mitragynine is one of the major constituent of Kratom, mitragynine is used as the marker compound for the identification and quantitation of Kratom in a variety of products. This Laboratory Information Bulletin describes methodology for the qualitative identification and quantitation of Kratom in different types of products such as but not limited to: powders, liquids, and spent-leaf materials. A quick methanolic based extraction procedure was used in combination with two instrument techniques: 1) GC/MS and/or LC-MS/MS for the initial screening and spectral confirmation of mitragynine in Kratom and quantitation via UPLC/PAD; 2) LC-MS/MS. Two different mass spectrometry systems were employed for confirmation/quantitation to permit flexibility within the regulatory laboratory for sample analysis. A mitragynine solvent standard was used for the comparative identification of Kratom and quantitation was reported based on the level of mitragynine in the product tested. Due to the low concentration of the mitragynine stock standard (100 µg/mL) and the high level of mitragynine in the products tested, traditional spiking of the standard via a wet/dry spike into a negative control was not feasible. Solvent based calibration curves were used for the quantitation of mitragynine in Kratom by UPLC/PDA and LC-MS/MS.

Validation was performed by characterizing a Kratom product purchased via the internet. This positive control was extracted seven times over three days and analyzed by all three analytical techniques: GC/MS, LC-MS/MS and UPLC/PDA. The UPLC/PDA data demonstrated a mean value of 1.041% (n=21, 4.2%) and the LC-MS/MS 1.140% (n=14, 6.81%) for mitragynine in the positive control. This positive control was extracted and analyzed in duplicate with every analytical batch.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   Yes.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   No, it only covers 1.0% mitragynine, and not the entire range in Table 1. Also the RSDr is > 3.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   Yes.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   No, it needs a safety statement.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   Yes, but they need to be clearer.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   no, not all of the parameters have been validated. The method only meets the > 0.5 - 15% range, nothing less.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   n/a, no CRM's listed for the plant product. Standard's only. One source of a standard is Chromadex.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   No, the LIB does not cover the other ranges as far as repeatability and recovery.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   Yes, there was no safety section.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   No, only mitragynine was analyzed in the "in-house" reference material. The mitragynine was quantitated by HPLC-DAD and not with the other techniques in the LIB. The material was not fully characterized.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   Yes and no, mitragynine was used solely. 7-OH mitragynine was not quantitated in the products.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes, it is fairly straight forward

5. *Based on the supporting information, what are pros/strengths of the method?*

   Multiple techniques, GC-MS, HPLC-PDAD, and HPLC-MS/MS. Uses small sample size. With more sensitive instruments that are now available, it would be possible to analyze at lower ranges.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   No data for the lower ranges. It is unsure if the LOD and LOQ have been met, since spikes were not done.

7. *Any general comments about the method?*

   It, basically, is a good method. It does need a lot of work before it becomes a first action method.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   no, it does not meet the SMPR requirements in table 1.

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: **Nour Eddine ES-SAFI**

Title of Method: **Quantification of Mitragynine in Kratom Raw Materials and Finished Products by High-Performance Liquid Chromatography: Singly Laboratory Validation**

AOAC Candidate Method Number**: KRA-02**

Applicable SMPR: **AOAC SMPR 2015.008**

I. SUMMARY OF METHOD

The method entitled "Quantification of Mitragynine in Kratom Raw Materials and Finished Products by High-Performance Liquid Chromatography: Singly Laboratory Validation" presents results dealing with the quantitative analysis of mitragynine in 8 matrixes including dried leaves, extract, capsule and beverages which were purchased from commercial vendors. The method used separation through an analytical HPLC with detection at 226 nm. Example of the obtained results showed the separation of various compounds with this method. The quantitative analysis of mitragynine in the studied samples was determined after calibration using a commercial mitragynine sample.

II. REVIEW OF THE METHOD ONLY:

1. Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.

The applicability of the method does not support the applicability of the SMPR. Thus, the **AOAC SMPR 2015.008** stated that "the Methods must be able to quantitate mitragynine, 7-hydroxymitragynine, and separate other relevant indole alkaloids of *Mitragyna speciosa*, in a broad range of matrices, including plant material, extracts, and finished products". The proposed KRA-02 method was tested on various different samples including dried leaves, extract, capsule and beverages. However, only mitragynine was quantified in the studied samples while the quantitative analysis of other analytes such as 7-hydroxymitragynine was not done The authors indicated that the content of 7-hydroxymitragynine was below the quantitation limit for all the explored samples, therefore this method is only valid for the detection and quantitation of mitragynine in raw materials, bulk extracts and finished products.

2. Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.

The analytical technique used in the method does not meet the SMPR.
Analytical range: not indicated
LOQ: as indicated (0.6 µg/mL) could not be compared to the SMPR values (% or ppm)
LOD: as indicated (0.2 µg/mL) could not be compared to the SMPR values (% or ppm)
Reproducibility: not indicated
Recovery: this was done only on a negative control sample. The obtained values were 105.2, 106.0 and 100.9 % for concentrations of the analyte of 0.5, 1.0 and 2.5 % respectively. The SMPR values indicated for this range are from 95 to 105 %. This shows that the first and the third value were out of the recommended ones.
Repeatability: According to the SMPR and taking into account the studied mitragynine concentration ranges, RSDr values should be ≤3 which is not the case of those given by the proposed method.

3. Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.

The definitions specified in the SMPR were generally used and applied appropriately in the method.

4. Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).

No precautionary or warning related to the method's reagents, components, instrumentation, or method steps were given.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.

The definitions specified in the SMPR were generally used and applied appropriately

2. Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.

As indicated above, the information given showed that the method does not meet the SMPR Method Performance Requirements table.

Analytical range: not indicated
Reproducibility: not indicated
Recovery: this was done only on a negative control sample. The obtained values obtained through KRA-02 method were 105.2, 106.0 and 100.9 % for concentrations of the analyte of 0.5, 1.0 and 2.5 % respectively. The SMPR values indicated for this range values ranging from 95 to 105 %. This shows that the first and the third value were out of the recommended ones.

Repeatability: According to the SMPR and taking into account the studied mitragynine concentration ranges, RSDr values should be ≤3 which is not the case of those given through the KRA-02 method.

3. Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.

A reference material (Mitragynine purchased from Chromadex and qualified using certified reference material from Cerilliant) was used for method recovery investigation. As indicated above, two of the three obtained values (105.2, 106.0 and 100.9) were out of the range indicated by the SMPR table (95-105 %).

4. Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.

For the 3 recovery given values, one (100.9 %) fit well in the range indicated in the SMPR table.

IV. GENERAL SUBMISSION PACKAGE:

1. Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?

The method could be assayed with a MS detection method

2. Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.

The method does not contain system suitability tests or controls as specified by the SMPR which should be done.

3. Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.

The following general comment is given in the conclusion section "The performance characteristics are within acceptable ranges according to AOAC Internatinal guidelines for dietary supplements" but no information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected is given.

4. Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.

The proposed KRA-02 is given such as a poster not a manuscript. Few details regarding the sample preparation, extraction, results and discussion are given.

5. Based on the supporting information, what are pros/strengths of the method?

Pros/strengths: Simplicity

6. Based on the supporting information, what are cons/weaknesses of the method?

Cons/weaknesses: detection method, only mitragynine is quantified

7. Any general comments about the method?

The proposed method is simple but may be critical on the fact of the detection at 226 nm which could, may be also detect other compounds than the target one. The other drawback of the proposed method is that it allows the quantification of only mitragynine.

V. RECOMMENDATION FOR THE METHOD:

Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

I do not recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL* taking into account the following points:

- The applicability of the proposed method does not support that of the SMPR
- The used detection method is not specific
- The method does not meet the minimum acceptance criteria given in the SMPR
- The method does not contain any system suitability tests and/or analytical quality control as specified in the SMPR.
- The method is not well written

Expert Review Panel - Method Review Form

Evaluation of Method # KRA-02

Title: Quantitation of Mitragynine in Kratom Raw Materials and Finished Products by HPLC Single Laboratory Validation

Author: Elizabeth Mudge

Summary of Method:

Reverse phase HPLC analysis with UV detection at 226 nm is used for quantitation of the Mitragynine content. Samples are extracted with 70 % aqueous methanol and subjected to a gradient HPLC analysis.

Method Scope/Applicability:

Well suited for a broad range of raw materials and finished products.

General comments about the method:

In general the method requires inexpensive HPLC equipment with minimal sample preparation. Needs more supporting data.

Method Clarity:

Sufficiently clear for use by technicians with minimal training.

Pros/Strengths:

•

Simple to use, relatively inexpensive equipment requirements. Easy sample prep. Relatively short analysis time.

Cons/Weaknesses:

Need more specific info on the data for precision, reproducibility, suitability. Reproducibility could be improved.
3.8 - 7.3 % reproducibility for powdered products.
4.5 - 5.7 % reproducibility for liquid products. I would expect better precision for the liquid products compared to the powders. Like to see some data on more aggressive extraction techinques (ie sonication, ultrasonic homogenization).

Supporting Data

- General Comment:

  Peak purity would be a useful parameter to include in the method.

  - Method Optimization:

    Extraction solvent study was nice. Perhaps some more data on the acid concentration since only the 0.1M concentration was graphed and the final assay is using 0.5 M.

    Since there is concern within regulatory agencies regarding the content of the 7-OH, some spiking recovery data would be useful for this compound.

- Performance Characteristics:

  Analytical Range:
  ~0.1 - 70 mg/g

  LOQ:
  0.6 ug / ml

  Accuracy/Recovery:
  100 - 106 %

  Precision (RSD_r):
  Not mentioned for each of the 3 days

  Reproducibility (RSD_R):
  3.8 - 7.3 %

- System suitability:

  Not mentioned in method

Recommendation:

Do you recommend that the ERP adopt this method as an AOAC Official Methods of Analysis (First Action status)?

Yes, pending additional method validation data. The author(s) mentioned that a manuscript for publication in the AOAC is being prepared, which hopefully will contain more method validation data for consideration. It would be beneficial if a draft of this document could be provided.

AFTER FIRST ACTION STATUS:

Are there any additional information that the ERP should consider in order to recommend the method for Final Action status?

Addition of quantitation of the 7-OH to the method since this compound is of specific interest to regulatory agencies.

Reviewer Name: Charles Metcalfe

Date : 11/30/15

Save Form

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Tom Phillips

Title of Method: Identification and Characterization of Indole and Oxindole Alkaloids from Leabes of Mitragyna speciosa Korth Using LC-Accurate QToF Mass Spectrometry

AOAC Candidate Method Number: KRA-03

Applicable SMPR : 2018.008

I. SUMMARY OF METHOD

The objective of this described work was to develop a single qualitative LC/quadrupole time of flight (QToF)-MS/MS method for the separation, characterization, and chemical profiling of alkaloids in association with chemometric analysis not only for assessing quality but also for the study of the variations in active constituents among samples of M. speciosa. Usually all alkaloids occur in multicomponent mixtures, and separation of these from other groups of compounds is the first requirementfor detailed structural analysis of alkaloids. This paper describes a method to resolve and characterize 12 indole and oxindole diastereomer alkaloids. The instrumentation consists of an ultra-HPLC (UHPLC) system coupled with a QToF mass spectrometer that can be used for chemical fingerprinting analysis of M. speciosa and is also suitable for the QC of various commercial samples. The fragmentation patterns for 7-hydroxymitragynine [1], isospeciofoline [2], isospeciofoleine [3], isorotundifoline [4], corynoxine B [5], corynoxine [6], 7β-hydroxy-7H-mitraciliatine [7], paynantheine [8], mitragynine [9], speciogynine [10], 3-isopaynantheine [11], and speciociliatine [12] were studied with proposed structures (Figure 1) for each significant product ion. With this characterization and chromatographic optimization, alkaloidal mixtures containing a large number of diastereoisomers were separated in extracts of M. speciosa leaves. The method offered more information about the chemical constituents of M. speciosa with the diastereomeric alkaloids identified and characterized according to retention times (RTs) and mass spectra.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   No, the method is for identification and structural elucidation only. There was not quantitation done.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   A qualified No. With more work it could have met the quantitation requirements.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   No, there was no discussion of supplements, LOD, LOQ, etc.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   No, there is no safety section.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   No, Please see above.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   No, there is not repeatability, recovery, LOD, or LOQ data presented. It is qualitative data only.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   No, no isotopically labeled standards were used.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   No, there is no quantitative data presented in the manuscript.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   No, there is no safety section.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   No, none were analyzed. The method is qualitative only.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   No, see above

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes, it is fairly straight forward.

5. *Based on the supporting information, what are pros/strengths of the method?*

   It uses a very selective and sensitive instrument, that can do quantitation as well as structural elucidation.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   No quantitation, no use of standards, etc.

7. *Any general comments about the method?*

   The method needs a lot of work to be advanced as first action.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   No, the method does not meet the SMPR requirements.

Evaluation of Method # KRA-03

Title: Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of *Mitragyna speciosa* Korth Using Liquid Chromatography-Accurate QToF Mass Spectrometry

Author: Bharathi Avula, Satyanarayanaraju Sagi, Yang-Hong Wang, Mei Wang, Zulfiqar Ali, and Troy J. Smillie

Summary of Method

Alkaloids were extracted from Mitragyna speciosa leaves & powders into methanol undergoing sonication and centrifugation. Extracts were filtered and injected onto a UHPLC/QToF-MS instrument. One sample, dried whole M. speciosa leaves, underwent additional sample preparation where the methanol extract was acidified, basified, and extracted into ethyl acetate. The solvent was evaporated and the extract dissolved in methanol prior to injection.
Separation was performed using a C8 column with water/acetonitrile gradient flow. The column was washed with acetonitrile between injections.
The parent and 3 to 5 fragment ions were used to characterize the chromatographic peaks and to then assess their presence in subsequent samples. Detailed evaluations of the mass spectra were described and used to create a spectral library for faster interpretation of data. Principle Component Analysis software was also used to assess the presence of M. speciosa and the potential for geographic and seasonal assessment of samples.

Method Scope/Applicability

The method identifies the presence of the 2 required compounds in the presence of 10 additional alkaloids found in M./ speciosa. The chromatographic and mass analysis protocols compliment each other very well, notably in differentiating between mitragynine and its diasteroisomers, speciogynine and speciociliatine.
The method does not quantitate either mitragynine or 7-hydroxymitragynine, the SMPR-required compounds. The other alkaloids were also not quantitated.

General Comments about the Method

This method as published has potential to qualitatively assess ingredients and extracts. The authors' evaluation of the large amounts of data and locating process to streamline the process should be commended.

Method Clarity

The method protocol and descriptions of data evaluation are clear and easily understood. The preliminary evaluation of PCA as a potential tool is intriguing and under development.

Pros/Strengths

The protocols for liquid chromatography and accurate mass analysis/interpretation are very sound and complete. They work together to assess the presence of relevant M. speciosa alkaloids.

Cons/Weaknesses

None of the alkaloids were quantitated using this method. The scope of this method may be able to be expanded to include quantitation because the reference standards were well characterized nd confirmed using TLC, HPLC, IR, 1D-NMR, 2D-NMR, and ESI-High Res-MS. The chromatographic and MS/MS conditions appear capable to allow expansion of the method's scope to include quantitation of the analytes.

Supporting Data

- **General Comment**
  N/A

  Method Optimization
  Method was optimized to qualitatively assess plant products and extracts for the presence of relevant alkaloids. No quantitation of these alkaloids were presented.

- **Performance Characteristics**

  Analytical Range:
  N/A

  LOQ:
  N/A

  Accuracy/Recovery:
  N/A

  Precision (RSD_r):
  N/A

  Reproducibility (RSD_R):
  N/A

System Suitibility:

N/A

Recommendation:
Do not recommend adopting this method as an AOAC OMA First Action. The method does not meet the SMPR requirements on quiantitating the relevant alkaloids.

Reviewer: John Szpylka
Date: 2 December 2015

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Christine R. Casey

Title of Method: Identification and Characterization of Indole and Oxindole Alkaloids from Leaves of Mitragyna speciosa Hroth Using Liquid Chromatography - Accurate QTof Mass Spectrometry

AOAC Candidate Method Number: KRA-03

Applicable SMPR : AOAC SMPR 2015.008

I. SUMMARY OF METHOD

The analytical method was developed to characterize and qualitatively determine the alkaloids from various M. speciose samples. The method uses two separate extraction procedures: a simple methanol extraction and an acid-base extraction procedure. Qualitative determination was performed via a RP C8 column with a water-acetonitrile formic acid mobile phase followed by high resolution mass spectrometry. The overall objective of the method was to develop a single qualitative LC-MS/MS (QTof)method for the separation, characterization, and chemical profiling of indoles and oxindole alkaloids in Mirtagynine speciosa.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   Overall, the method does not meet all the requirements to support the AOAC SMPR 2015.008. Presently, the method does not preform quantitation for 7-hydroxy mitragynine, mitragynine, system suitability, repeatability, and reproducibility. However, the method does separate 7-hydroxy mitragynine, mitragynine, and other relevant indoles of Mitragynine speciose.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   The analytical technique does separate and confirm identity of 7-hydroxy mitragynine, mitragynine, and other relevant indoles of Mitragynine speciose, as stated in question 1. The method is not quantitative per the AOAC SMPR 2015.008.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   Most of the definitions specified in the SMPR are appropriate in this method. Since the method is not quantitative specific definition did not apply for this method such as repeatability and reproducibility.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   Yes

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   There is no information demonstrating the requirements in table 1. Again, the method is qualitative be design hence, the parameters for the single-laboratory validation does not apply. The method stills perform well for the stated purpose of the qualitative work. The method could be quantitative with the addition of a calibration curve.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   The method does use reference materials. Due to the expertise of the authors in natural product analysis, the researchers were able to isolate individual indoles and oxindole. This

enabled the researchers to determine retention times and to insure the separation of pertinent indole alkaloids.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   The requirements in table 1 are for the application of quantitative analysis. As stated earlier, this method is qualitative in nature and does not contain the data stated in Table 1. The method would be easily modified to cover the requirements in Table 1, such as system suitability, calibration curve, determination of LOD and LOQ, recovery, and repeatability.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   No

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   The method does contain control sample and individual components specified by the SMPR. As a qualitative method, the method demonstrates the performance for the identification and confirmation for 7-hydroxy mitragynine, mitragynine, and other revenant indole alkaloids.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   The controls used in the method worked appropriately and as expected. The major components of Mitragynine speciose was identified and separated via LC-MS/MS (QTof).

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes, the method is very well written and easy to follow.

5. *Based on the supporting information, what are pros/strengths of the method?*

   There are many strengths to this method, such as the able to separate and confirm the identity of indole and oxindole by liquid chromatography - high resolution mass spectrometry, isolation of approximately 11 indole alkaloids not presently available by commercial vendors, and the chemometric analysis of the data generated. The method can be easily modified to include the requirements of Table 1 single-laboratory validation for a more quantitative.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

The method does not contain the method performance requirements stated in the AOAC SMPR 2015.008 single laboratory requirements. As stated earlier, the method is qualitative but the requirements are for a quantitative method. The method can be modified to include a calibration curve and the requirements in table 1 could be meet. One significant drawback is the ability of other laboratories to perform the method due to the requirement of a QTof or Orbitap. If a laboratory could demonstrate the separation, maybe the method could be transferred to a PDA.

7. *Any general comments about the method?*

Overall, this method addresses the main issue of analytical separation and identification of 7-hydroxy mitragynine, mitragynine, and 11 other indole alkaloids. If quantitation is important for the SMPR 2015.008, the method can be modified.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

I would overall recommend this method with the few comments I have mentioned in the above sections. Per the AOAC SMPR 2015.008, Table 1method performance requirements, the method would require some additional analysis to determine the analytical range , LOD, LOQ, recovery, and repeatability.

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Yan-Hong Wang

Title of Method: LC/MS Method for the Identification of Mitragyna speciose (Kratom) and Quantitation of Mitragynine Using Linear Ion Trap Mass Spectrometer

AOAC Candidate Method Number: KRA-04

Applicable SMPR :

I. SUMMARY OF METHOD

An LC/MS method is developed for quantitative analysis of mitragynine and 7-hydroxymitragynine. The method has been applied to liquid drinks, liquid tinctures, powders, bulk ground processed leaves, dried leaves and capsules.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   The method isn't validated for the parameters precision. The linear range of calibration curve is not specified.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   Yes.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   The determination of LOD and LOQ is not specified in the method.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   The method, as written, does not contain all appropriate precautionary and warning.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   The determination of precision, recovery, LOD and LOQ is not specified in the method.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   The method doesn't explain the determination of precision, recovery, preparation of standard solution, and linear range of calibration curve.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify what is missing and how this impacts demonstration of performance of the method.*

   Reference Material hasn't been specified in the method.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   Validation of this method is not clearly demonstrated.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

The method should be validated for precision, LOD and LOQ.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   Yes.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   Yes.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   The information including preparation of standard solution, spiking, calibration curve, and precision is missing from this method.

5. *Based on the supporting information, what are pros/strengths of the method?*

   This method can identify multi alkaloids in Mitragyna speciosa and related samples, and is useful for the quantitative determination of mytragynine and 7-hydroxymitragynine.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   This method is not completely validated.

7. *Any general comments about the method?*

   The developed method is suitable for the analysis of mitragynine and 7-hydroxymitragynine in Mitragyna speciosa and related products.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   This method should be further validated.

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Catherine (Kate) Rimmer

Title of Method: LC/MS Method for the Identification of Mitragyna speciose (Kratom) and Quantitation of Mitragynine Using Linear Ion Trap Mass Spectrometer

AOAC Candidate Method Number: KRA-04

Applicable SMPR : SMPR 2015.008

I. SUMMARY OF METHOD

This method is an LC/MS method for the determination of mitragynine mass fraction and the identification of Mitragyna speciosa. For the identification several of the alkaloids are separated.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   Yes

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   Yes, all methods are acceptable for the SMPR.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

Definitions are not provided, but they way the terms are used appears to be correct.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   No safety notes are provided

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   They appear to be, but the method is very short.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   Anatlytical range not specified for either alkaloid, LOQ not specified for either alkaloid. LOD for both alkaloids meet SMPR. No spike recovery for 7-OH, spike recovery for Mitragynine is 72-145 % (SMPR is 95-105%, does not meet requirement), Repeatability not reported for either alkaloid. Reproducibility reported as "less than 4%" for mitragynine (Meets SMPR), not reported for 7-OH.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   A biological reference material extract was used as a positive control

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   Outlined in section 2

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   There are no precautionary steps. Usual lab precautions should be sufficient.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   No suitability requirements. A biological control material was used.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   No, the control was not used for quantitative methods and there were no system suitability requirements

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes. It is clear and concise (maybe too concise)

5. *Based on the supporting information, what are pros/strengths of the method?*

   Good chromatography, a nice range of samples were tested

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   An internal standard would help. Also, it was not fully validated for both alkaloids

7. *Any general comments about the method?*

   The method would be greatly improved with the use of a labeled internal standard. Also, the lack of information about the 7-OH alkaloid needs to be addressed

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   Not at this time, if further information supporting the analytical range, LOQ, LOD, recovery, RSDr, and RSDR is added for both analytes of interest, then I would consider it (although the spike recovery is out of range and would likely be improved by the use of labeled internal standards)

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MEMORANDUM

DATE: **DECEMBER 10, 2015**

TO: **SPDS SET 2 EXPERT REVIEW PANEL MEMBERS (ASHWAGANDHA)**

FROM: **AOAC INTERNATIONAL**

SUBJECT: **ASHWAGANDHA METHOD SUBMISSIONS AND REVIEWS**

BACKGROUND:

One method was submitted in response to the Ashwagandha Call for Methods. The reviews submitted are provided in this meeting book, and links to the *Standard Method Performance Requirements*[SM] (SMPRs) and the candidate methods themselves are provided below.

AOAC Candidate Method Number	Submitter	Primary Reviewer	Secondary Reviewer(s)
ASH-01	Natural Remedies	Kurt Young	Anton Bzhelyansky; Prashant Ingle

SMPRs:

- AOAC SMPR 2015.007: *Standard Method Performance Requirements* for Withanolide Glycosides and Aglycones of Ashwagandha

Expert Review Panel π aŜűK2Ř wŜØ⁄ŜØ C2ℕY

Evaluation of Method # AHH-01

Title: Estimation of Withanolides (Withanoside IV, Withaferin A, 12-Deoxywithastromonolide, Withanolide A, Withanolide B) in Withania somnifera

Author: Rojison Koshy, Sr. Research Officer Natural Remedies, Private Limited; Validation Report author

Summary of Method:

Compounds specific to Withania somnifera (Ashwaganda) (Withanolides) are extracted from sample matrices with methanol and analyzed against a mixture of external standards using reverse phase HPLC with UV detection at 227 nm.

Method Scope/Applicability:

The method is applicable to analysis of raw root and extracts of root material from Withania somnifera and to products containing these materials along with excipients.

General comments about the method:

This method appears to have sufficient validation for intended use. One major issue is that this method as written is evidently a precursor to the more simplified method that appears in the USP monographs for Ashwaganda and Ashwaganda Powdered Extract.

Method Clarity:

The method clearly outlines the procedures for calibration, system suitability, chromatographic system setup, sample preparation and results calculations.

Pros/Strengths:

•

Pros: Uses readily available instrumentation and reagents. Calibration and results calculations are simple using external standards. Component chromatographic peaks are sufficiently separated in sample and standard chromatograms.

Cons/Weaknesses:

Cons: Using external standards for each analyte will be very expensive. The multiple extraction steps followed by solvent concentration are unnecessary for extracts and products with extracts. Older, less efficient columns are used which leads to long run times. Several withanolides listed in the USP monograph and in subsequent literature do not appear in this method.

Supporting Data

- General Comment:

  Sufficiant validation data was provided, which included specificity, reproducibility, linearity, accuracy, ruggedness and sample and standard solution stability

  - Method Optimization:

    No method optimization data was provided in addition to the validation report data.

- Performance Characteristics:

  Analytical Range:

  ~18.7-1321 ug/mL

  LOQ:

  Not given. Lowest levels for individual standards for linearity study were 18.7-23.0 ug/mL.

  Accuracy/Recovery:

  Withanoside IV: 94.6-101.8%, Withanoside V: 95.3-101.2%, Withaferin A: 95.9-100.7%, 12-Deoxy-Withanstramonolide: 91.2-101.6%, Withanolide A: 93.7-99.6%, Withanolide B: 74.0-106.0%

  Precision (RSD_r):

  From validation summary report: Withanoside IV: 0.5%, Withanoside V: 0.86%, Withaferin A: 0.5%, 12-Deoxy-Withanstramonolide: 0.6%, Withanolide A: 1.5%, Withanolide B: 0.9% (Note: the criteria for Precision in the Summary Report does not match that in the body of the report.)

  Reproducibility (RSD_R):

  Reported as 5.0% overall in validation summary- no ranges given for different levels.

- System suitability:

  Resolution >3 for Withanoside V and Withaferin A. Analyte peak tailing factors <1.5. Precision of injection <2.5.

Recommendation:

52 è2dz NŜŎ2YYŜyŘ űKI·ű űKŜ 9wt I·Ř2LJű űKλă YŜűK2Ř I·ă I·y´ ! h ! / hŦŦλŎλI·f aŜűK2Řă 2Ŧ ! yI·fèăλă óCΛNăű ! ŎűΛ2y´ăűI·údzăúK

I do not recommend adopting this method as written. However, I would recommend adopting an updated method with simplified standard preparations and simplified sample extraction for powdered extracts per the USP monograph method.

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I recommend looking over the USP monographs and any additional references that may elucidate additional withanolides, especially those that may be extracted in an aqueous environment. Also, I recommend seeking out information on the utility of this method for use with extracts from other parts of the plant.

wŜØλŜøŜN bI·YŜY Kurt Young

5I·űŜY 12/07/15

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Method Review Form - AOAC INTERNATIONAL Standards Development

Name of Reviewer: Anton Bzhelyansky

Title of Method: Withanolide Glycosides and Aglycones of Ashwagandha (*W. somnifera*)

AOAC Candidate Method Number: ASH-01

Applicable SMPR : 2015.007

I. SUMMARY OF METHOD

Withanolides in ashwagandha raw material and various extracts are determined following methanolic extraction and using an acetonitrile gradient against phosphate buffer on a conventional octadecylsilyl column, with UV detection at 227 nm.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   Method does not explicitly state LOQs for individual withabolides. Through the back-of-the-envelope calculations, I arrived at about 5 µg/mL for aglycones, and about 15 µg/mL for glycosides. It may, however, be mentioned that the 10-ppm (0.001%) LOQ may be considered unnecessarily low, considering that ashwagandha root typically contains 0.25 – 0.35% of withanolides. Besides, a cursory look at the very busy chromatographic baseline of the plant at 227 nm would defy determination of withanolides at the concentrations specified in SMPR; a similar exercise with the standards will be just a purely analytical exercise. The degree of granularity requested in the SMPR Table 2, for accuracy, repeatability and reproducibility at four different levels was likewise not addressed in method validation, but is likely not necessary.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   HPLC is likely to be a technique of choice for fulfilling the requirements of this SMPR, save for, perhaps, CE. Therefore, the chosen methodology is appropriate.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   SMPR definitions are utilized in the method appropriately; they are rather universal, and are not misinterpreted in any way.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   No specific precautions should be required; these are routine stock analytical procedures.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   SMPR definitions are utilized in the method appropriately.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   Please see II.1. I don't believe that incomplete adherence to all the requirements of the SMPR document negatively affects method applicability. This is a good mature method; at least 8 years of continuous use.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   The presented method, and the accompanying validation, utilizes six reference materials: Withanoside IV, Withanoside V, Withaferin A, 12-Deoxywithastramonolide, Withanolide A and Withanolide B. The SMPR document lists ten potential reference material candidates. In this reviewer's opinion, six reference standards are likely excessive for this method. My recommendation would be to use one RM to account for withanolide glycosides and one for withanolide aglycones. Theoretically, even a single RM could be utilized provided that RRFs are calculated appropriately.

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

Method developers demonstrate applicability for analysis of *Withania* raw material (powdered root), and three types of extracts: aqueous, hydroalcoholic and methanolic. SMPR in addition requests analysis of unspecified finished dosage forms. On iherb.com about half of the finished products are simple capsules, with about 15% tablets. There are no grounds to expect degradation of method performance in solid finished dosage forms. Performance with respect to liquid extracts and softgels may be additionally examined, as they would likely necessitate an adjustment to sample preparation. Still, even without these additional matrices, the existing procedure will address about 80% of the materials currently present in the US market.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   This is a rather awkwardly worded question. The techniques specified in the method are conventional lab prep work, and they do not merit specific precautionary statements.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   Method presents a more extensive set of System Suitability tests, relevant to the specific analytical task:

   a. System precision NMT 2.5% for all components of the standard solution.
   b. Resolution NLT 3.0 between Withanoside V and Withaferin A (Note: this will require these analytes to be available to method practitioners). For comic relief, the analytes are listed as ephedrine and pseudoephedrine.
   c. Tailing NMT 1.5% for all components of the standard solution.
   d. Linear regression coefficient NLT 0.998 for all components of the standard solution in successive dilutions.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   SMPR specifies blanks and periodic check standards throughout the run. The use of bracketing standards is a routine practice and there is no evidence that this method will not support it. The use of blanks, especially periodic, should, in my opinion be well-justified – e.g., when carryover is suspected. There is no evidence suggesting that carryover may be an issue. Furthermore, the use of check standards is generally guided by the individual laboratory's quality systems and may not necessarily belong in the method. I believe that this may need to be justified if recommended. I, however, would suggest to adopt the System suitability parameters suggested by the method submitter as more immediately relevant.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Method is put together very well. The instructions are clear and well thought-out. The amount of details supplied is adequate. It is, however, not obvious whether it is suggested to prepare numerous dilutions of the standard: on the one hand, correlation coefficient is supplied in

System suitability and the System precision is proposed for the 'level 4' dilution of the standard; on the other, the Standard preparation procedure does not include any instructions on preparation of a series of solutions. Given the demonstrated linearity of the method, I would favor a single-point standard with the number of components possibly less than the suggested six: perhaps, one glycoside and one aglycone.

5. *Based on the supporting information, what are pros/strengths of the method?*

   Method has been thoroughly validated, it represents a result of procedure in continuous use, perfected over time. Instructions are clearly written, most details have been worked out well.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   Require fewer standards – analysis will be too expensive and laborious.
   Provide RRFs for other components.
   Single-point standard concentration may be preferable.
   Investigate other ashwagandha chemotypes – this plant is notorious for that.
   Peak integration might be a challenge for less experienced chemists. However, peak purity is superb for such a complicated chromatogram.
   Consider faster chromatography even with the obvious sample complexity.
   Following sample filtration, indicate the volume to be discarded if any.
   Include anti-adulteration provisions – measures to detect admixture of adulterants/confounders, and possibly chromatographic ID of different plant parts utilized.

7. *Any general comments about the method?*

   Method validation is done thoroughly; it adequately examines method performance. Despite minor departures from the SMPR, it is deemed adequate for analysis of *W. somnifera* samples; including raw materials and finished dosage forms. Clarifications regarding utilization of reference materials, preparation of standard solutions and system suitability tests should be sought from the method submitters. Generally, I would recommend adoption of the method without major modifications.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   Yes.

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MEMORANDUM

DATE: **DECEMBER 10, 2015**

TO: **SPDS SET 2 EXPERT REVIEW PANEL MEMBERS (FOLIN C)**

FROM: **AOAC INTERNATIONAL**

SUBJECT: **FOLIN C METHOD SUBMISSIONS AND REVIEWS**

BACKGROUND:

Three methods were submitted in response to the Folin C Call for Methods. The reviews submitted are provided in this meeting book, and links to the *Standard Method Performance Requirements*[SM] (SMPRs) and the candidate methods themselves are provided below.

AOAC Candidate Method Number	Submitter	Primary Reviewer	Secondary Reviewer(s)
FOL-01	University of Scranton	John Finley	Jungmin Lee
FOL-02	OmniActives	Tom Phillips	Aniko Solyom
FOL-03	University of Istanbul	Joseph Zhou	Dana Krueger

SMPRs:

- AOAC SMPR 2015.009 - Standard Method Performance Requirements[SM] for Estimation of Total Phenolic Content Using the Folin-C Assay

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: John Finley

Title of Method: Folin-Ciocalteau

AOAC Candidate Method Number: FOL-01

Applicable SMPR : SMPR 2015-009

I. SUMMARY OF METHOD

Single and dual reagent methods are described in detail and compared.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*
   The method appears to meet the SMPR goals. The advantage of determining both free and bound phenolics and options for removal or interferences.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*
   Yes.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*
   Yes.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*
   Yes.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   The method is described in a previous publication. No significant issues differ from the SMPR

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   Performance criteria are adequately covered

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   Yes, the authors show examples in a variety of extracts and preparations

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   Yes the range of materials tested covers a wide spectrum

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   No.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   Yes.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   Yes, they could have demonstrated a broader range of standards.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes, well written and easily followed.

5. *Based on the supporting information, what are pros/strengths of the method?*

   Method illustrates applicability of=ver wa wide range of test materials and includes clean up procedures to remove interferences.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

   Method is clearly described, would be useful in a range of materials and allows a range of test procedures form single reagent or dual reagent to microplate.

7. *Any general comments about the method?*

   Method is useful over a broad range of testing. Better description of appropriate standards would be beneficial.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   Yes.

Expert Review Panel - Method Review Form

Evaluation of Method # AHH-01

Title: Estimation of Withanolides (Withanoside IV, Withaferin A, 12-Deoxywithastromonolide, Withanolide A, Withanolide B) in Withania somnifera

Author: Rojison Koshy, Sr. Research Officer Natural Remedies, Private Limited; Validation Report author

Summary of Method:

Compounds specific to Withania somnifera (Ashwaganda) (Withanolides) are extracted from sample matrices with methanol and analyzed against a mixture of external standards using reverse phase HPLC with UV detection at 227 nm.

Method Scope/Applicability:

The method is applicable to analysis of raw root and extracts of root material from Withania somnifera and to products containing these materials along with excipients.

General comments about the method:

This method appears to have sufficient validation for intended use. One major issue is that this method as written is evidently a precursor to the more simplified method that appears in the USP monographs for Ashwaganda and Ashwaganda Powdered Extract.

Method Clarity:

The method clearly outlines the procedures for calibration, system suitability, chromatographic system setup, sample preparation and results calculations.

Pros/Strengths:

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Pros: Uses readily available instrumentation and reagents. Calibration and results calculations are simple using external standards. Component chromatographic peaks are sufficiently separated in sample and standard chromatograms.

Cons/Weaknesses:

Cons: Using external standards for each analyte will be very expensive. The multiple extraction steps followed by solvent concentration are unnecessary for extracts and products with extracts. Older, less efficient columns are used which leads to long run times. Several withanolides listed in the USP monograph and in subsequent literature do not appear in this method.

Supporting Data

- General Comment:

  Sufficiant validation data was provided, which included specificity, reproducibility, linearity, accuracy, ruggedness and sample and standard solution stability

  - Method Optimization:

    No method optimization data was provided in addition to the validation report data.

- Performance Characteristics:

  Analytical Range:

  ~18.7-1321 ug/mL

  LOQ:

  Not given. Lowest levels for individual standards for linearity study were 18.7-23.0 ug/mL.

  Accuracy/Recovery:

  Withanoside IV: 94.6-101.8%, Withanoside V: 95.3-101.2%, Withaferin A: 95.9-100.7%, 12-Deoxy-Withanstramonolide: 91.2-101.6%, Withanolide A: 93.7-99.6%, Withanolide B: 74.0-106.0%

  Precision (RSD_r):

  From validation summary report: Withanoside IV: 0.5%, Withanoside V: 0.86%, Withaferin A: 0.5%, 12-Deoxy-Withanstramonolide: 0.6%, Withanolide A: 1.5%, Withanolide B: 0.9% (Note: the criteria for Precision in the Summary Report does not match that in the body of the report.)

  Reproducibility (RSD_R):

  Reported as 5.0% overall in validation summary- no ranges given for different levels.

- System suitability:

  Resolution >3 for Withanoside V and Withaferin A. Analyte peak tailing factors <1.5. Precision of injection <2.5.

Recommendation:

Do you recommend that the ERP adopt this method as an AOAC Official Methods of Analysis (First Action status)?

I do not recommend adopting this method as written. However, I would recommend adopting an updated method with simplified standard preparations and simplified sample extraction for powdered extracts per the USP monograph method.

AFTER FIRST ACTION STATUS:

Are there any additional information that the ERP should consider in order to recommend the method for Final Action status?

I recommend looking over the USP monographs and any additional references that may elucidate additional withanolides, especially those that may be extracted in an aqueous environment. Also, I recommend seeking out information on the utility of this method for use with extracts from other parts of the plant.

Reviewer Name: Kurt Young

Date : 12/07/15

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Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Tom Phillips

Title of Method: Method for the Estimation of Total Phenolic Content using the Folin-C Assay

AOAC Candidate Method Number: FOL-02

Applicable SMPR : 2015.008

I. SUMMARY OF METHOD

Phenolics include simple phenols, phenolic acids (benzoic and cinnamic acid derivatives), coumarins, flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins. These compounds are among the most widely occurring secondary metabolites in the plant kingdom, acting mainly as phytoalexins, attractants for pollinators, and contributors to plant pigmentation, antioxidants, and protective agents against UV light, among others. Although quantitative determination of polyphenols is hampered by their structural complexity and diversity, several methods have been used to determine polyphenols in plant extracts. Assuming that quantification of individual polyphenols does not adequately reveal the proportion of polymeric procyanidins, and then spectrophotometry in the ultraviolet region may be a useful tool to help resolve this problem. Colorimetric reactions are widely used in the UV/VIS spectrophotometric method, which is easy to perform, rapid, reproducible, reliable and applicable in routine laboratory use, and low-cost. However, it is important that colorimetric assay need to use a reference substance, and then this method measures the total concentration of phenolic hydroxyl groups in the plant extract against reference standard. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteu's phenol reagent) to form a blue complex that can be quantified by visible-light spectrophotometry. The reaction forms a blue chromophore constituted by a phosphotungstic-phosphormolybdenum complex, where the maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds.The reaction generally provides accurate and specific data for several groups of phenolic compounds.

II. REVIEW OF THE METHOD ONLY:

1. *Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.*

   No, the method that was submitted was for only one product. The applicability is for any dietary supplement raw material or finished products.

2. *Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.*

   Yes.

3. *Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.*

   No, LOQ was done with standards and not in matrix. Only one ingredient was studied.

4. *Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).*

   No, there is no safety section.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. *Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.*

   There was no supporting documentation submitted. Therefore, it was unable to be reviewed.

2. *Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.*

   Yes, it was very minimal. Only one matrix studied, not enough replicates and/or days per Appendix K. The method as written is good for only one set of species, not the wide range of dietary supplements. It also would have an impact upon the reproducibility and repeatability of the method.

3. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.*

   No, there were no reference materials used in the validation. This impacts the accuracy of the method in that only spikes were used and not a reference material with incurred phenolics. The assumption is that the sample was spiked with Gallic Acid, and this leads to the question, what about the other types of phenolics present in dietary supplements? Would the incurred phenolics actually be measured?

4. *Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.*

   Yes, but it is very limited. Only one type of plant was studied, and only and extract. Not all the forms of the supplements were studied.

IV. GENERAL SUBMISSION PACKAGE:

1. *Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?*

   Yes, there was no safety section in the method.

2. *Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.*

   No, the check standards are missing, as well as the matrix interferents study.

3. *Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.*

   No, Please see #2.

4. *Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.*

   Yes, it is fairly straight forward. The calculations need to be explained further, along with the use of the linear curve to calculate the result, instead of a single point calibration.

5. *Based on the supporting information, what are pros/strengths of the method?*

   The strengths of the method is that it is fairly straight forward. The throughput of the method should be high, since the time per sample is fairly short.

6. *Based on the supporting information, what are cons/weaknesses of the method?*

Weaknesses are not enough matrices studied. Sodium carbonate solution does not last very long.

7. *Any general comments about the method?*

   It needs a lot of work, especially extra matrices.

V. RECOMMENDATION FOR THE METHOD:

1. Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

   At this point, it cannot be recommended for First Action.

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Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Aniko M. Solyom

Title of Method: Method for the estimation of total phenolic content using the Folin-C assay

AOAC Candidate Method Number: FOL-02

Applicable SMPR: AOAC SMPR 2015.009

I. SUMMARY OF METHOD

"Standard" F-C method to estimate the phenolic content in Salacia species.

II. REVIEW OF THE METHOD ONLY:

1. Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.

   The method was applied to estimate the phenolic content in one type of raw material and extract, but there is no data for finished products.

2. Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.

   Yes, it is a standard Folin-C method, without any significant modification.

3. Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.

   Details are missing from the method:
   1. How LOQ was calculated, based on standard deviation of the blank, signal-to-noise ratio, standard deviation of the response and the slope, or any other method?
   2. What was the timeframe during the repeatibility studies?
   3. How the recovery studies were performed: exhaustive extraction of the plant material before adding the standards? The recovery data table is not quite clear.

4. Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).

There are no precautions and warnings included in the method.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.

The supporting documentation did not answer the questions mentioned above.

2. Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.

   Analytical range: SMPR 5-500 ppm; Method 5-100 ppm
   Limit of Quantitation: SMPR < 5 ppm; Method not clear how it was calculated
   Recovery and Repeatibility: Not clear how they were calculated

3. Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify the what is missing and how this impacts demonstration of performance of the method.

None of the Reference Materials stated in the SMPR were used.

4. Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.

The method was used to estimate the total phenolic content only in one species

(Salacia), powdered sample and extract. There is no data for dietary supplement finished products.

IV. GENERAL SUBMISSION PACKAGE:

1. Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?

2. Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.

   No. The method indicates using a blank sample, but no data regarding the blank is reported. There is no optimization data; for example investigation of the effect of the time for color development.

3. Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.

   There is no system suitability test data.

4. Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.

   The method is written as a standard SOP for a specific product.

5. Based on the supporting information, what are pros/strengths of the method?

   Simple, "standard" Folin-C method, most likely works well for the specified product.

6. Based on the supporting information, what are cons/weaknesses of the method?

There is no data showing that the method can be applied to other products than S

7. Any general comments about the method?

There are couple of spelling errors (decent vs decant, Abosrabance vs. Absorbar

V. RECOMMENDATION FOR THE METHOD:

Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.

I would not recommend the method, because in this form it is applicable only to one specific plant. Different matrices and possible interfering compounds were not evaluated.

Method Review Form

AOAC INTERNATIONAL Standards Development

Name of Reviewer: Joseph Zhou

Title of Method: Modified Folin−Ciocalteu Antioxidant Capacity Assay for Measuring Lipophilic Antioxidants

AOAC Candidate Method Number: FOL-03

Applicable SMPR :

I. SUMMARY OF METHOD

This is a modified Folin-Ciocalteu method that is able to measure both hydrophilic and lipophilic antioxidants.

II. REVIEW OF THE METHOD ONLY:

1. ***Does the applicability of the method support the applicability of the SMPR? If no, please explain what is missing.***

   This method does not meet the SMPR: 1) The method is for the measurement of total antioxidant capacity, not total phenolic content; 2)Analytical range is shorter; 3)No RSDr data at low concentration < 5ppm; 4) No RSDR data.;

2. ***Does the analytical technique(s) used in the method meet the SMPR? If no, please specify what how it differs from what is stated in the SMPR.***

   I think this is a very good method, but some data as required in the SMPR are not available.

3. ***Are the definitions specified in the SMPR used and applied appropriately in the method? If no, please indicate how the terms are used.***

   The definition specified in the SMPR are used and applied in the method, but the data are incomplete.

4. ***Does the method, as written, contain all appropriate precautionary and warning related to the method's reagents, components, instrumentation, or method steps that may be hazardous? If no, please suggest wording or option(s).***

   It is a research article. This method as is does not meet the SMPR. No precautionary and warning were mentioned.

III. REVIEW OF INFORMATION IN SUPPORT OF THE METHOD:

1. ***Are the definitions specified in the SMPR used and applied appropriately in the supporting documentation (manuscripts, method studies, etc…)? If no, please explain differences and if the method is impacted by the difference.***

   The definition specified in the SMPR are used and applied in the method, but the data are incomplete.

2. ***Is there information demonstrating that the method meets the SMPR Method Performance Requirements table? If no, for any of the parameters in the SMPR Method Performance Requirements table, then please explain what is missing and the impact on performance of the method.***

   The method meets the SMPR in the following areas: 1) LOQ < 5ppm (0.75ppm for trolox); 2)Recovery 80-110% (101% for trolox); 3)RSDr <7% when the concentration >5mmp (RSDr = 4% when the concentration =15ppm). But this method does not meet the SMPR in the following areas: 1) The method is for the measurement of total antioxidant capacity, not total phenolic content; 2)Analytical range is shorter; 3)No RSDr data at low concentration < 5ppm; 4) No RSDR data.

3. ***Is there information demonstrating that the method performs within the SMPR Method Performance Requirements using the Reference Materials stated in the SMPR? If no, then specify what is missing and how this impacts demonstration of performance of the method.***

   No reference materials are specified in this SMPR. The method performance is shown above.

4. ***Is there information demonstrating that the method performs within the SMPR Method Performance Requirements table specifications for all analytes in the SMPR applicability statement? If no, please specify what is missing and whether or not the method's applicability should be modified.***

The method performance is shown in the answer to question 2 above. The SMPR does not specify any analytes in the statement, but this paper indicates that the method works for a lot of analytes although for some analytes, the method 's applicability does need to be modified.

IV. GENERAL SUBMISSION PACKAGE:

1. ***Based on the supporting information, were there any additional steps in the evaluation of the method that indicated the need for any additional precautionary statements in the method?***

   Yes, if this method is chosen as the best method, it should add a precautionary statement to indicate the danger (safety) of the chemical solvents used in the method.

2. ***Does the method contain system suitability tests or controls as specified by the SMPR? If no, please indicate if there is a need for such tests or controls and which ones.***

   This is a classic method, a blank test was used in the method development work.

3. ***Is there information demonstrating that the method system suitability tests and controls as specified in the SMPR worked appropriately and as expected? If no, please specify.***

   This method indicated a good system suitability and control test.

4. ***Based on the supporting information, is the method written clearly and concisely? If no, please specify the needed revisions.***

   This method was presented in a research paper format. If it is chosen as the best one, this method needs to be rewritten in the AOAC method formate.

5. ***Based on the supporting information, what are pros/strengths of the method?***

   This research paper presented a revised Folin-C method that is capable to test both hydrophilic and lipophilic antioxidants in a sample. However the original Folin-C method can only test hydrophilic contents in a sample. Therefore this revised Folin-C method is more accurate in the estimation of total polyphenolic contents in a sample. In addition, this method was successfully applied to test many popular raw materials that contain higher polyphenolic contents (the FOL-02 presented in this ERP review only tested one compound although it is written in a right format.)

6. ***Based on the supporting information, what are cons/weaknesses of the method?***

   The data is incomplete. If this method is chosen as the best one, there is a need to do a SLV on the method .

7. ***Any general comments about the method?***

This is a very good research paper that indicated an important improvement of the original Folin-C method. The original Folin-C method is good at testing hydrophilic antioxidant capacity of a sample. However the revised method presented in this paper, if the data presented are reliable, can test both hydrophilic and lipophilic antioxidant capacity of a sample.This is an important improvement in the method accuracy. However a SLV is required to 1) validate the method; and 2) obtain additional data as required in the SMPR to support the method.su

V. RECOMMENDATION FOR THE METHOD:

1. **Do you recommend this method be adopted as a First Action and published in the *Official Methods of Analysis of AOAC INTERNATIONAL?* If no, please specify rationale.**

This method is not ready to be adopted as a First Action although it is likely to have a better accuracy in estimation of the total antioxidant capacity of a sample. An SLV is required to validate the method.

Expert Review Panel π aŜűK2Ř wŜØ⁄ŜØ C2ℕY

Evaluation of Method # AHH-01

Title: Estimation of Withanolides (Withanoside IV, Withaferin A, 12-Deoxywithastromonolide, Withanolide A, Withanolide B) in Withania somnifera

Author: Rojison Koshy, Sr. Research Officer Natural Remedies, Private Limited; Validation Report author

Summary of Method:

Compounds specific to Withania somnifera (Ashwaganda) (Withanolides) are extracted from sample matrices with methanol and analyzed against a mixture of external standards using reverse phase HPLC with UV detection at 227 nm.

Method Scope/Applicability:

The method is applicable to analysis of raw root and extracts of root material from Withania somnifera and to products containing these materials along with excipients.

General comments about the method:

This method appears to have sufficient validation for intended use. One major issue is that this method as written is evidently a precursor to the more simplified method that appears in the USP monographs for Ashwaganda and Ashwaganda Powdered Extract.

Method Clarity:

The method clearly outlines the procedures for calibration, system suitability, chromatographic system setup, sample preparation and results calculations.

Pros/Strengths:

•

Pros: Uses readily available instrumentation and reagents. Calibration and results calculations are simple using external standards. Component chromatographic peaks are sufficiently separated in sample and standard chromatograms.

Cons/Weaknesses:

Cons: Using external standards for each analyte will be very expensive. The multiple extraction steps followed by solvent concentration are unnecessary for extracts and products with extracts. Older, less efficient columns are used which leads to long run times. Several withanolides listed in the USP monograph and in subsequent literature do not appear in this method.

Supporting Data

- General Comment:

  Sufficiant validation data was provided, which included specificity, reproducibility, linearity, accuracy, ruggedness and sample and standard solution stability

  - Method Optimization:

    No method optimization data was provided in addition to the validation report data.

- Performance Characteristics:

  Analytical Range:

  ~18.7-1321 ug/mL

  LOQ:

  Not given. Lowest levels for individual standards for linearity study were 18.7-23.0 ug/mL.

  Accuracy/Recovery:

  Withanoside IV: 94.6-101.8%, Withanoside V: 95.3-101.2%, Withaferin A: 95.9-100.7%, 12-Deoxy-Withanstramonolide: 91.2-101.6%, Withanolide A: 93.7-99.6%, Withanolide B: 74.0-106.0%

  Precision (RSD_r):

  From validation summary report: Withanoside IV: 0.5%, Withanoside V: 0.86%, Withaferin A: 0.5%, 12-Deoxy-Withanstramonolide: 0.6%, Withanolide A: 1.5%, Withanolide B: 0.9% (Note: the criteria for Precision in the Summary Report does not match that in the body of the report.)

  Reproducibility (RSD_R):

  Reported as 5.0% overall in validation summary- no ranges given for different levels.

- System suitability:

  Resolution >3 for Withanoside V and Withaferin A. Analyte peak tailing factors <1.5. Precision of injection <2.5.

Recommendation:

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I do not recommend adopting this method as written. However, I would recommend adopting an updated method with simplified standard preparations and simplified sample extraction for powdered extracts per the USP monograph method.

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I recommend looking over the USP monographs and any additional references that may elucidate additional withanolides, especially those that may be extracted in an aqueous environment. Also, I recommend seeking out information on the utility of this method for use with extracts from other parts of the plant.

wŜØλŜøŜN bI·YŜY Kurt Young

5I·űŜY 12/07/15

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