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Current initiatives for the validation of analytical methods for botanicals☆

Paula N Brown and Patience Lister

The demand for validated analytical methods for botanicals has grown in response to the increasing consumer market for botanical supplements. Government initiatives to increase the availability of validated analytical methods and botanical reference material have led to the publication of numerous validation studies in scientific journals. Single laboratory validation and collaborative validation studies are structured to confirm a method's ruggedness and fit for purpose. The performance characteristics and statistical protocols followed throughout a validation study vary with the source of guidelines. Analytical techniques and priority methods are influenced by the need for fast-screening techniques, the limited availability of reference material, market value, and the prevalence of contaminants in botanical supplements.

Addresses
Natural Health and Food Products Research Group, British Columbia Institute of Technology, 3700 Willingdon Avenue, Burnaby, British Columbia V5G 3H2, Canada

Corresponding author: Brown, Paula N. (pbrown@bcit.ca, Paula_Brown@bcit.ca)

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Introduction

A growing consumer market for botanical supplements has surpassed the availability of reliable analytical methods to verify botanical identify, purity and strength. The lack of publicly available validated methods makes it difficult to assess product quality, both composition and stability, and has stymied scientific research on these products. This for validated methods is further driven by laws that require publicly available methods to enforce legal action against dietary supplements [1].

Initiatives have been taken in response to the need for validated analytical methods for botanical supplements. These involve collaborations between government, industry and private scientific organizations where scientists and industry members have been working to develop and validate standard analytical methods for dietary supplements [1]. Despite the U.S. Food and Drug Administration's (FDA) Current Good Manufacturing Practices (CGMP) for dietary supplements, the industry still suffers from botanical misidentification, product contamination and adulteration. The National Institutes of Health's (NIH) initiative to validate methods for priority dietary supplements drove AOAC International to adapt the traditional Official Methods process to include single laboratory validation (SLV). The scope of this review includes initiatives, guidance and current practice in the validation of analytical methods for botanicals.

Validation

Validation is an applied approach to verifying that a method is suitable and rugged enough to function as a quality control tool. AOAC International defines a validated method as a method that is fit for its intended purpose. The purpose may include quantifying a specific analyte in a product, confirming whether a product meets its specifications or regulations, identifying the presence of a nutrient or contaminant in a product, or identifying a product ingredient. Methods can be validated in a single laboratory or through a collaborative study in multiple laboratories (AOAC guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals; URL: http://www.aoac.org/vmeth/SLV_Guidelines_Dietary%20Supplements.pdf).

Single laboratory validation

A Single Laboratory Validation (SLV) is the first step towards becoming an official method of analysis (OMA) through AOAC International. Once the method has passed a SLV, it is ready for a collaborative study between multiple laboratories. If the collaborative study is successful then the method may be considered for an OMA.

An analytical method should be fully developed and optimized before single laboratory validation. The purpose of the validation is to confirm performance parameters determined during development and should provide information on how it will perform under routine use. An unstable method may require re-validation [2]. If validation results do not meet the performance standards then the method may require further development and

optimization. When possible, a validation should also be conducted as a collaborative study by multiple laboratories, on different instruments, reagents, and standards.

Collaborative study

The purpose of a collaborative study is to determine the reproducibility of performance characteristics when followed by different laboratories. Under AOAC International guidelines, this requires a minimum of 10 independent laboratories producing valid data for 12 replicates of each material. All samples are blinded and randomized [1]. Some methods are validated independently through small-scale inter-laboratory studies [3]. This can provide information on method ruggedness but will not lead to an OMA. A 20 laboratory collaborative study was recently conducted on the analysis of the mycotoxin ochratoxin A in licorice products. The method was considered successful based on meeting the LOD set out in EU legislation [4•].

Reference material

Chemical analysis requires reference points. Analytical methods for botanicals typically require reference materials with measurable physical properties that are used for comparison to the test materials. Chemical standards are a common form of reference material that can be purchased from chemical suppliers; however, if no reference material exists then a compound with similar properties can be used. As part of method development, reference materials should be assessed for identity, purity, stability, and storage conditions [1]. Botanical identification methods (BIM) require that the availability and identity of panel materials be verified [5••]. Some reference standards can be isolated in-house, as was done with baicalein-7-O-glucoside for the analysis of *Semen oroxyli*. Authors checked its purity by UV and NMR spectroscopy [6•].

Botanical reference materials, or voucher specimens, are preserved specimens that can be used to authenticate sample identification and can be sourced from herbariums [7,8]. When botanical material is collected from wild sources, a voucher specimen should be verified and saved for future reference [9]. Germplasm banks are another potential source of verified plant material [10•].

Performance characteristics

The performance characteristics of an analytical method include applicability (scope), selectivity, calibration, accuracy, repeatability precision, measurement uncertainty, variability, limit of detection (LOD), and limit of quantification (LOQ). They indicate the degree to which replicate measurements approach the 'true' values of a method's parameters. Other characteristics that should be measured during the method development and optimization stage include analyte stability, matrix effects, sensitivity, and ruggedness or robustness [1,9].

According to AOAC International, a chemical calibration curve should have six or more calibration points that span the relevant range. This practice was followed using six calibration levels ranging in concentration of 10–215 μg/kg for validating a method for quantifying deoxynivalenol-3-glucoside in processed cereal products [12]. An assessment was done to confirm the stability of cichoric acid during its extraction and analysis from Echinacea. This involved an exhaustive extraction procedure, storage at room temperature for six days, and 30 consecutive HPLC injections [13•].

Ruggedness is the degree to which a method's results can be reproduced under different conditions. The Youden Ruggedness Trial is a statistical tool used to identify how significantly each factor contributes to a method's variability. It involves small, deliberate changes to the procedure and then an assessment of the results [1]. The Youden Ruggedness Trial was used to examine the effect of seven parameters of an extraction method for cranberry anthocyanins. High and low parameters were examined for sample mass, sonication time, percent acid in extraction solvent, shaking time, sonication temperature, injection time, and centrifugation time [14••].

Statistical tools

Current practices are now shifting over to design of experiment (DOE) for evaluating ruggedness. DOE is considered a time-efficient and cost-efficient technique used to simultaneously identify the effects of multiple factors on results.

Chemometrics is gaining significance as a data analysis tool and can be used for method development by identifying the effects of analytical conditions in factorial experiments. This tool was used in combination with DOE for the analysis of alkaloids in poppy straw. A 24 full factorial design was accomplished through 19 GC/FID/MS method optimization experiments. Using DOE, the authors were able to identify the most effective parameters for rapid screening [15••]. Some authors forgo statistical methods for evaluating ruggedness and instead vary parameters individually to see which significantly affect results [16,17,19]. This can be a time consuming approach.

The Horwitz ratio (HorRat) is a statistical performance parameter that indicates the acceptability of method precision. It is a common criterion for validation of analytical methods under AOAC International protocols and has an acceptable value range of 0.3–1.3 for SLV data and 0.5-2.0 for collaborative study data (Definitions and

calculations of HorRat values from intralaboratory data; URL: http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/HORRAT_SLV.pdf). HorRat was a useful indicator of good overall method performance of a validated method for the analysis for Catechin and Epicatechin in cocoa [19].

Leading organizations and government bodies

The ODS is a key government leader in the development and validation of analytical methods and reference materials for botanicals and dietary supplements. Goals of the ODS's Analytical Methods/Reference Materials Dietary Supplements Program (AMRM) are to build infrastructure for validation, support the development of validated methods, reference material, and calibration standards, and make validated methods and reference materials available to the community (Analytical methods/reference materials (AMRM) dietary supplements program description; URL: http://ods.od.nih.gov/Research/AMRMProgramDescription.aspx).

AOAC International is a not-for profit organization standards body that develops program foundation and technical aspects of analytical methods. They coordinate scientific studies, evaluate results, give official sanction to applicable methods, and disseminate the methods or performance data to the public (Help the dietary supplement community create a reliable compendium of collaborative studied methods; URL: http://www.aoac.org/dietsupp6/Dietary-Supplement-web-site/DSHomePage2.html). The AOAC's Presidential Task Force on Dietary Supplements identifies priority ingredients and selects methods for validation. They rank ingredients based on funding, market share of the ingredient, availability of methods to validate, related clinical trials, and ingredient safety concerns. AOAC's expert review panel (ERP) assists in this process by peer-reviewing the quality of existing methods and supporting data. The ERP recently reviewed methods for antioxidant activity of botanicals. They were evaluated against AOAC International's standard method performance requirements (SMPRs) and an ORAC method was approved as First Action Official Method [20].

Other organizations that provide SLV guidance include International Conference on Harmonization (ICH) and Brazil's Agencia Nacional de Vigilancia sanitaria (ANVISA) [21,22].

Priority methods and botanicals

The ODS publishes a list of ODS-supported validated methods and botanical ingredients. Methods that are currently under development and awaiting validation include those for black cohosh, general botanicals, glycosides, pesticides, and valerian (Analytical Methods for Dietary Supplements; URL: http://ods.od.nih.gov/Research/AMRMAnalyticalMethods.aspx#umethods). AOAC's ERP prioritizes methods and analytes of interest for validation. In 2009 the ERP selected pyridoxine as the main analyte of interest after reviewing over 50 vitamin B6 methods [23••].

Ginseng is one of the top selling dietary supplements on the market and has been assessed in various clinical trials. This led to the execution of SLV and interlaboratory studies of an accurate and reliable method to identify and quantify ginsenosides in different ginseng species [24••,25•]. This method is now on tract to becoming an OMA by AOAC Int. OMAs can be extended to the analysis of new matrices, but should be validated to confirm applicability. Authors extended AOAC's Official Method 991.31 for the determination of Aflatoxins in corn, raw peanuts, and peanut butter to analysis of aflatoxins in botanical roots [26•].

Pesticide analysis is an important aspect of identifying contaminants and adulterants in botanicals. Although matrix effects pose challenges, the high presence of pesticides in botanicals has led to validation studies [27,28,29•]. The growing consumer interest in gluten-free foods has also driven the need to validate analytical test methods for gluten [30•].

Standardization of plant material is a critical aspect of botanical supplement quality. As a first step towards standardizing oenothein B in plants from the Oenotheraceae family, authors developed and validated a HPLC–DAD-MS method for its quantification [31]. In response to the FDA's request that supplement manufacturers adopt new technologies for measuring process attributes, authors validated a NIR method for quantification of chlorogenic acid in *Lonicera japonica* [32]. Interest in the bioavailability of botanical compounds has led to the validation of analytical methods on biological matrices, such as plasma [33]. In preparation for a Phase I clinical trial, a UHPLC/MS/MS method was validated for the quantification of prenylflavonoids in human serum. The method provides an accurate and precise way to measure clinical samples and support product efficacy [39••].

Analytical techniques

Fast screening techniques and simple sample preparation are growing priorities intended to make methods more convenient and cost-effective [34•]. Authors combined this need with the interest in alkaloid analysis to develop and validate a rapid-screening method for simultaneous alkaloid analysis in poppy straw [35]. An HPTLC-densitometric method was developed and validated to meet the need for a high throughput procedure for routine quantification of primulasaponins [36•]. Derivatization procedures, which can increase sample preparation or

run times, are being replaced by faster techniques when possible [37•].

Another motivation for increasing the efficiency of analytical methods is to conserve plant material. A 6-min UHPLC–QTOF-MS method was developed and validated for high-throughput analysis of huperzine A in a species of club moss. The authors validated the microscale extraction protocol and short analytical method with the goal of conserving plant resources [38•].

Conclusion

This review shows that the increasing consumer use of botanical supplements has led to initiatives and support for validating analytical methods to verify their identity, purity and strength. Through government, academic and industry initiatives, validated analytical methods and reliable reference material are becoming more prevalent and available to the public. Fast screening techniques and efficient use of limited study material are priorities in method design and optimization. Commercial value as well as the prevalence of pesticides and allergens in botanicals is leading the direction of validated analytical methods.

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