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Simple Authentication Methods for Herbal Ingredient Integrity in the face of EMA

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Ingredient Identification

- Identity determination is integral to cGMPs.
- "Identity tests for botanical ingredients should be specific enough to distinguish the correct plant species and plant part(s) from known and potential adulterants. Identity testing is usually based on the form of the ingredient at the time of testing, i.e., whether the herb is in whole, cut, powdered, or extracted form."

Bilberry Extract Authentication

- AHPA problem, tools, solution approach
  - Anthocyanins turn blue at elevated pH
  - HPTLC produces characteristic information
  - AHP and USP monograph standards
  - *J. Agric. Food Chem.* 2004;52(4):688 -691
    - Nature's Sunshine, HPLC-MS
  - *J AOAC Int.* 2007;90(4):911-919
    - Indena, HPLC-UV

American**Herbal Products** Association

Resource**LINKS**

Introduction

The American Herbal Products Association is providing here analytical tools and methods to identify adulteration of powdered raw materials labeled as bilberry (*Vaccinium myrtillus*) extract. There are well-established scientifically valid methods for determining the presence of red dye in materials purported to be powdered bilberry extracts. They are provided now as immediately available practical tools for industry in order to deal appropriately with reports of adulteration of this ingredient and make wise purchasing decisions.

"Bilberry" Extracts Adulterated with Artificial Dyes

In the process of evaluating commercial material labeled as bilberry extract, AHPA member company MediHerb investigated a sample that appeared to meet the listed specification for anthocyanin content by simple spectrometry. However upon further analysis it was discovered that this material was not likely derived from bilberry and was in fact adulterated with amaranth dye (red dye no. 2). Their work has since been published (see: Bilberry adulteration using the food dye amaranth. Penman KG, Halstead CW, Matthias A, De Voss JJ, Stuthe JM, Bone KM, Lehmann RP. *J Agric Food Chem.* 2006 Sep 20;54(19):7378-82).

Subsequent to the work by Penman *et al*., Steven Dentali, PhD, AHPA's vice president of scientific and technical affairs, authored an article published in the trade journal *Nutraceuticals World*, which can be accessed ***here***. This article warns that lower price material available from nontraditional sources can be an initial indication of an adulterated extract. This is especially problematic where common methods of analysis are easily "cheated" so that a specification is met, but the material is not what it should be.

The information presented here is intended to educate industry about the potential quality problem with ingredients labeled as bilberry extract and to provide a scientifically valid method to aid in the differentiation between genuine bilberry fruit extract and material adulterated with amaranth dye. When examined with these methods by qualified individuals, information should be obtained that will allow more informed purchasing decisions than may be available with single wavelength spectroscopic analysis.

Methods for Detection of Dye in "Bilberry" Extracts

Anthocyanins turn blue at elevated pH

Bilberry extracts are blue-black in color but form a pink solution when diluted at neutral pH. At elevated pH, anthocyanins change color and anthocyanin-containing solutions, such as can be made by diluting bilberry extract with water, turn blue. This is illustrated ***here*** where the far left solution is very dilute true bilberry extract, next is this solution with the pH adjusted to greater than 10, next is dilute fake bilberry extract under the same conditions, and lastly very dilute fake bilberry extract.

This experiment is simple to replicate with bilberry extract or any other anthocyanin-containing materials and so is not necessarily indicative of bilberry anthocyanins. In fact this principle is easily observable by adding baking soda to dilute anthocyanin containing solutions. A "kitchen test" using diluted red wine, or grape juice, or other suitable (anthocyanin-containing) juice can be performed to illustrate this property of anthocyanins. Sprinkling baking soda into such a prepared dilute reddish solution will cause it to turn purple or bluish. This same color transformation will happen with bilberry (or other anthocyanin containing) extract, but does not occur with amaranth dye.

High performance thin layer chromatography (HPTLC)

Authentic bilberry can be easily differentiated from materials adulterated with amaranth dye under the HPTLC conditions used to create the image shown ***here***. Lanes 1 and 4 are authentic bilberry extract. The horizontal colored bands represent anthocyanin compounds. The adulterated samples in lanes 2 and 3 have a different pattern of colored bands and, most notably, a bright red band that corresponds to amaranth dye. The last lane shows what pure amaranth dye looks under these same conditions.

Click ***here*** for the HPTLC conditions used to produce this result.

Other Relevant Sources of Bilberry Information

AHP and USP monographs

The American Herbal Pharmacopoeia and Therapeutic Compendium has published a bilberry fruit monograph that includes standards of analysis, quality control, and therapeutic information. More information can be found at ***http://www.herbal-ahp.org/titles.htm***.

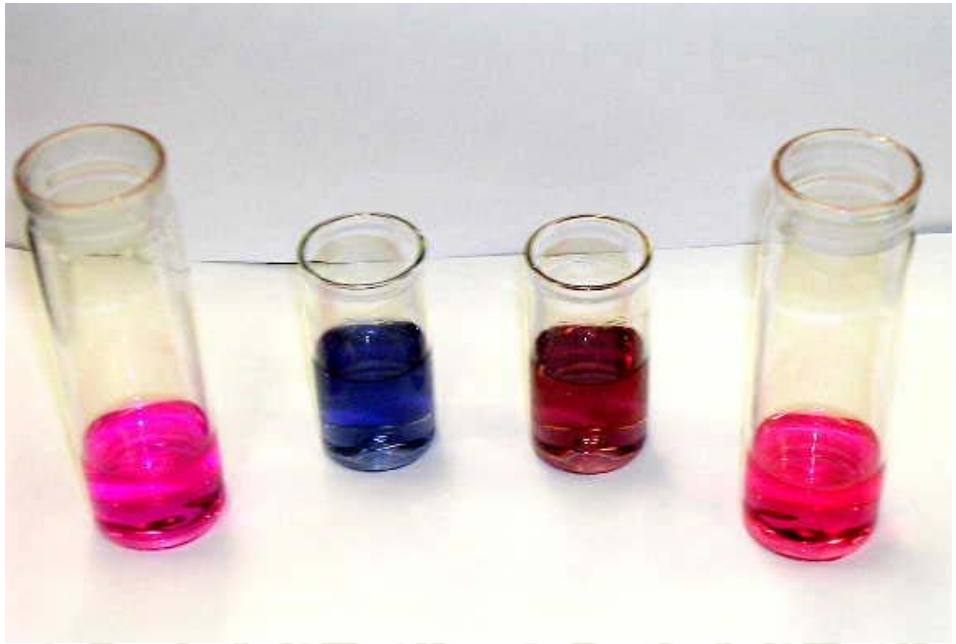
The United States Pharmacopeia has published a dietary supplement monograph for powdered bilberry extract as an in-process revision in the *Pharmacopeial Forum,* July–Aug. 2007;33(4):685-688.

Other compositional analysis and quantification of bilberry anthocyanins

The Journal of Agricultural and Food Chemistry has published a method of anthocyanin analysis in bilberry. That abstract can be accessed ***here***.

A more recent paper published by the *Journal of AOAC INTERNATIONAL* "was developed and validated for the identification and quantification of both anthocyanins and anthocyanidins present in bilberry extracts and products." The abstract for it is available ***here***.

Anthocyanin Color Change

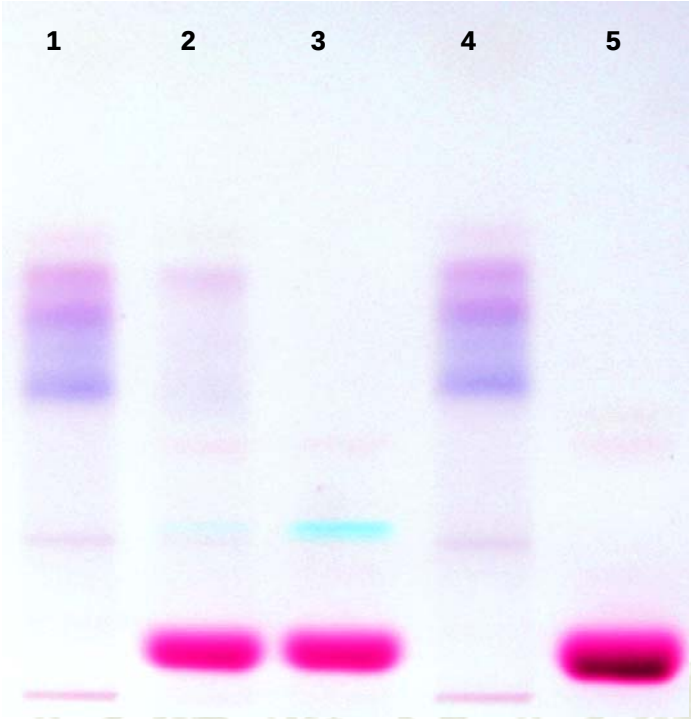


Left to Right: Very dilute Bilberry, Bilberry adjusted to pH>10, Fake Bilberry adjusted to pH>10, and Fake very dilute Bilberry

Bilberry HPTLC Conditions

- Sample preparation
  - Approximately 20mg of powdered sample was mixed with 1 mL methanol, then
  - sonicated for 10 min and centrifuged.
- Sample application
  - 2 μ L of samples were are applied as 7mm bands, 3mm apart and 10mm from lower edge of plate with a CAMAG Linomat IV applicator
- Plate: Merck HPTLC 10x10 cm, Si 60 F254
- Tank: Twin Trough Chamber
- Saturation: 30 min with filter paper
- Developing distance from application position/lower edge: ~70 mm
- Developing solvent: 5 ml of 1-Butanol, water, formic acid (40:15:10)
- Plate drying: oven
- Visualization: back illumination with white light

Bilberry Extract HPTLC



Left to Right: Lane 1 genuine bilberry, lane 2 and 3 fake bilberry, lane 4 genuine bilberry, lane 5 pure adulterant (i.e., amaranth dye)

(Photos courtesy of Medi-Herb, Palmyra, WI)

Powdered *Hoodia gordonii* stems

- Problem: substitution
  - Other species and inert materials
- Tools
  - Microscopy, HPTLC, HPLC
- Solution
  - Intelligent application of the tools with knowledge of their strengths and limitations

Hoodia gordonii

Introduction — The American Herbal Products Association is providing here analytical tools for identification and determination of purity of powdered raw materials labeled as *Hoodia gordonii* stems. The work was commissioned by the AHPA *Hoodia gordonii* Committee and managed by AHPA staff. It was initiated through solicitation of proposals that were evaluated by an independent expert scientific review panel.

The tools consist of three methods for the characterization and identification of material labeled as *Hoodia gordonii* stem. These are microscopy, high-performance thin layer chromatography (HPTLC), and high-performance liquid chromatography (HPLC). These techniques are complementary tools to provide information regarding identity and purity of raw powdered material, and should be employed in concert.

Microscopy — When employed by a qualified microscopist trained in analyzing botanical powders, this technique is capable of revealing the presence of cell components that are unique to a test material as well as negative markers (i.e., cell structures that do not occur in a test material).

The images provided here show characteristics of authentic material, but microscopic features indicative only of *Hoodia gordonii* stem have not yet been found. So far microscopy cannot reliably differentiate *Hoodia gordonii* from related *Hoodia* species or from *Caralluma fimbriata*. However, the presence of calcium oxalate crystals, and specific cellular substructures known as sclereids and stone cells always indicate the presence of material that is NOT from *Hoodia* species.

While not capable of positively identifying *Hoodia gordonii* stem by itself, microscopy is nevertheless very useful for detecting certain added adulterants. Microscopy is able to reveal the presence of *Opuntia* spp. added to *Hoodia gordonii* powder at a 1% concentration. Similarly the presence of maltodextrin can be detected reliably at 10% adulteration and possibly even at 1% and 5% concentrations.

> Click here for **Microscopy images and a technique to detect adulteration with maltodextrin or *Opuntia* spp.**
> *Note: Not all web browsers present this linked page properly. Internet Explorer is among those that does show the page.*

HPTLC — When properly employed, High Performance Thin Layer Chromatography (HPTLC) provides a visual display of compounds present in test materials. However, this does not necessarily mean that HPTLC will always provide authentication to the exclusion of related species and all possible adulterants.

The HPTLC method used here is a very powerful tool for identification of the presence of *Hoodia gordonii* stem based on the characteristic image produced. The extent of variability of results for authentic materials has not yet been determined, nor has the extent of HPTLC to detect mixtures of authentic material with added adulterants.

> Click here for an **HPTLC method for *Hoodia gordonii***

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Click here for an **HPTLC method for *Hoodia gordonii***

HPLC — Recent advances in chromatography has made the High Performance Liquid Chromatography (HPLC) very useful for the analysis of targeted analytes in complex matrices such as botanical-based dietary supplements. The rigour and confidence in an analytical result is determined by a number of key factors including the competence of the analyst, the method selection and appropriateness, reference materials employed, and finally the test material itself. Two main approaches may be employed for HPLC analysis. They are marker or active determination, and profiling (often referred to as "fingerprinting") techniques.

While it may sometimes be preferable to quantify a specified constituent or class of compounds, the selection of analytes and methods of analysis can be driven by marketing, ease of analysis, or other relatively arbitrary reasons. It is very important when selecting or developing an analytical method to carefully consider, "Will the question being asked be answered by the analysis results?" Always use analytical methods within their specified scope and applicability. For *Hoodia gordonii* the question was simply, "Is this material *Hoodia gordonii* or not?"

The question of identity is actually the hardest one to answer by any analytical technique. To definitively answer it there must be diagnostic features such as morphological, chemical, or genetic that can be used to differentiate *Hoodia gordonii* from related species and known adulterants. Certain identified marker compounds known as oxypregnane glycosides have been employed to determine the presence of *Hoodia* species. Although the best known of these, P57, has been used as a marker for *Hoodia gordonii* it is not necessarily diagnostic for this species alone because P57 can be found in other *Hoodia* species. Furthermore, published HPLC methods of analysis for P57 can be confounded by co-eluting peaks that are found in adulterating species. For example the mistaken assignment of P57 in *Gymnema sylvestre* can occur if the HPLC conditions are not optimized for this problem as demonstrated in **Figure 1**.

Click here for an **HPLC method for *Hoodia gordonii***

A recent publication by Avula *et al*. reported similar findings. Lengthening the HPLC run time for this method (see **Figure 2**) now separates the peak in *Gymnema sylvestre*, as shown in **Figure 3**, which previously corresponded to that of P57 with the unmodified method. The better optimized method clearly reveals 11 oxypregnane glycosides from *Hoodia gordonii* as shown in **Figure 4**. **Figure 5** demonstrates the wide variety of HPLC "fingerprints" that can be generated for *Hoodia gordonii*. Future work may entail the acquisition of additional samples so that a wider chemical representation of *Hoodia gordonii* materials can be subjected to this evaluation. Discrimination from other potentially adulterating species, such as *Stapelia* spp., will be aided by their evaluation with this method and comparison of the results.

Click here for a HPTLC image that clearly differentiates authentic *Hoodia gordonii* material from *Opuntia ficus-indica*.

HPTLC Technical Details

Sample preparation
Mix 0.5 g of powdered sample with 5 mL methanol or methanol/water 8:2, then sonicate for 10 min and centrifuge.

Standard preparation
Dissolve 7 mg of fructose in 5 mL of ethanol/water 7:3
Dissolve 2 mg of beta-sitosterol in 5 mL of methanol
Dissolve 0.6 mg of P57 in 2 mL of methanol

Application
Apply 5 µL of the prepared samples and standards as 8 mm wide bands, which should be at least 2 mm apart and 8 mm from the lower edge of the plate.

Other Technical Details
Temperature: 28°C
Humidity: 49%
Plate: Merck HPTLC glass 20x10 cm, Si 60 F_{254} batch HX 745979
Tank: twin trough chamber 20x10 cm
Saturation: 20 min with filter paper
Developing distance from application position/lower edge: 62/70 mm
Developing solvent: chloroform/methanol/water 70:30:3
Developing time: 10 min
Plate drying: 5 min in a stream of cold air

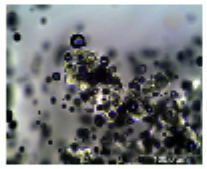
Visualization
Derivitizing agent: anisaldehyde
Preparation: 10 mL sulfuric acid is carefully added to an ice-cooled mixture of 170 mL methanol and 20 mL acetic acid, before adding 1 mL anisaldehyde
Application: the plate is dipped then heated at 100°C for 3 min

Equipment
CAMAG Automatic TLC Sampler 4, CAMAG Automatic Developing Chamber (ADC2) with humidity control ($MgCl_2$ to RH 33%), CAMAG Chromatogram Immersion Device III, CAMAG TLC Plate Heater III, CAMAG Digistore 2, and CAMAG filter paper for chamber saturation base ADC2, IKA Mill KB5/10, Hettich Centrifuge EBA21, Telsonic Ultrasonic Bath TPC25, and Mettler-Toledo DC4400 and AG245 Balances.

Technique to detect adulteration of *Hoodia gordonii* powder with maltodextrin or *Opuntia* spp.

- Use a small spatula to place about 5-10 mg of powdered sample on a clean microscope slide.
- Add 3 drops of acidified chloral hydrate-glycerol reagent solution to the slide making sure the sample is sufficiently surrounded. Then add 1-2 more drops directly on top of the sample.
- Place a clean cover slip over the sample by lowering an edge of it close to the edge of the reagent sample mixture and then lower the rest of the cover slip over the remaining area of the reagent sample mixture using the first placed edge as a hinge.
- Immediately analyze the prepared slide under the microscope with at least 200X magnification.

Detection of maltodextrin or other soluble excipients.

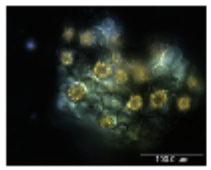


While it is normal for some plant material to effervece upon contact with acidified chloral hydrate-glycerol solution, dried plant material does not normally dissolve. Dark material effervescing and melting (see image to left) into a clear or slightly pigmented solution is indicative of non-plant material. It only takes seconds for this to occur so multiple attempts may be required.

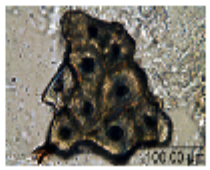
Detection of *Opuntia spp.*

Once the analysis for the detection of maltodextrin is complete, place the prepared slide on a hot plate set at low-medium heat until a gentle boil is produced. Remove the prepared slide and allow it to cool to room temperature before reanalyzing with at least 200X magnification.

The presence of rosettes or prisms of calcium oxalate crystals, sclereids, or stone cells is significant as they are not found in *Hoodia gordonii.* They are present in *Opuntia* spp. and their presence in material labeled as *Hoodia gordonii* indicates the presence of *Opuntia* spp. or other non-*Hoodia gordonii* material.



Rosettes



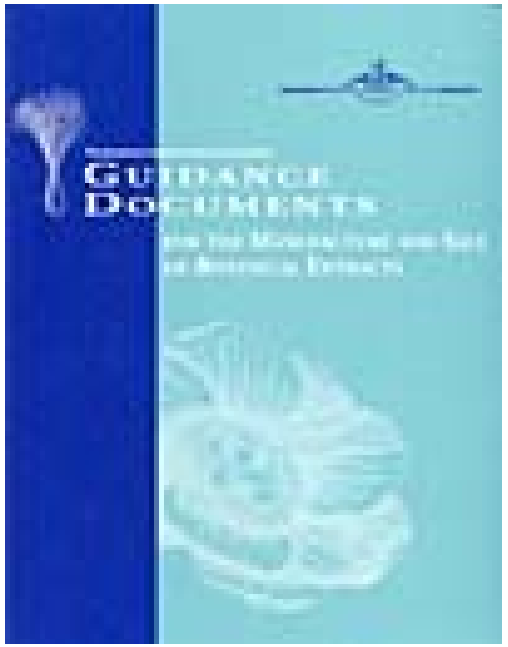
Stone Cells

Approach Aspects

- Educational
  - Visually accessible to nonscientist
  - Persistent web presence
- Practical
  - Scientifically valid but not validated
  - QC ready adoptable methods
- Implementation
  - Simple starting point immediately practical

AHPA Guidances

- *Guidance Documents for the Manufacture and Sale of Botanical Extracts*
  - ***Standardization of Botanical Products***
  - ***Use of Marker Compounds in Manufacturing and Labeling Botanically Derived Dietary Supplements***



THANK YOU!

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