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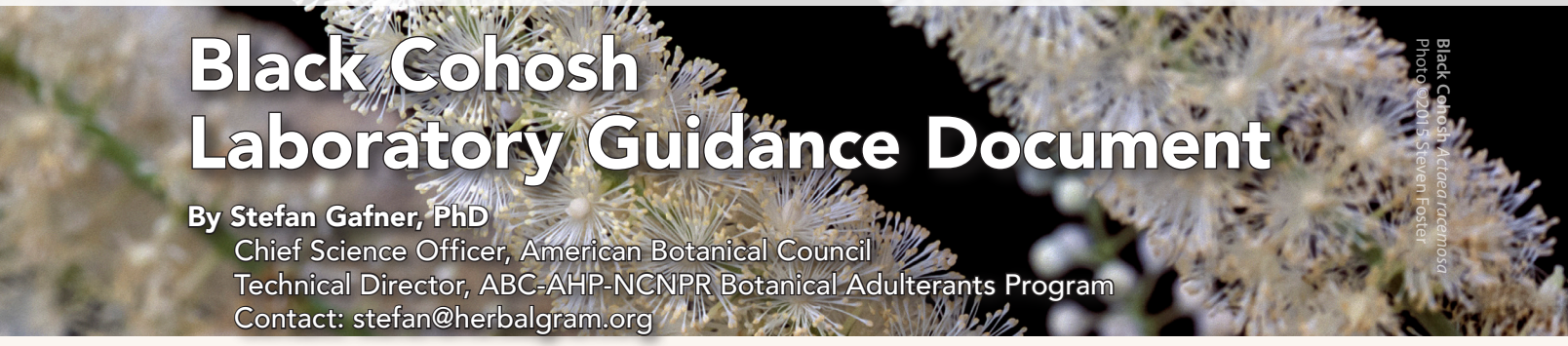
Botanical Adulterants Program
American Botanical Council • the American Herbal Pharmacopoeia • the University of Mississippi's National Center for Natural Products Research

Black Cohosh Laboratory Guidance Document


Black Cohosh *Actaea racemosa*
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1. Purpose

In recent years, adulteration of black cohosh (*Actaea racemosa*, Ranunculaceae) roots and rhizomes has become more apparent. Adulteration predominantly occurs with Chinese species of *Actaea* such as *A. heracleifolia*, *A. dahurica*, and *A. cimicifuga* (all known by the common name Chinese cimicifuga and by the Chinese name of *sheng ma*). Additionally, the Chinese cimicifuga (*sheng ma*) market is commonly adulterated with *Serratula chinensis* (*guang dong sheng ma* [Asteraceae]). Adulteration has also been reported with North American *Actaea* species growing in the same area as black cohosh, such as *A. pachypoda*, *A. rubra*, and *A. podocarpa*. This Laboratory Guidance Document presents a review of the various analytical technologies used to differentiate between authentic *A. racemosa* and its potentially adulterating species.

2. Scope

The various analytical methods were reviewed with the specific purpose of identifying strengths and limitations of the existing methods for differentiating *A. racemosa* from its potentially adulterating species. Analysts can use this review to help guide the appropriate choice of techniques for their specific black cohosh products for qualitative purposes. The recommendation of a specific method for testing *A. racemosa* materials in their particular matrix in this Laboratory Guidance Document does not reduce or remove the responsibility of laboratory personnel to demonstrate adequate method performance in their own laboratories using accepted protocols outlined in the United States Food and Drug Administration's Good Manufacturing Practices (GMPs) rule (21 CFR Part 111) and those published by AOAC International, International Organization for Standardization (ISO), World Health Organization (WHO), and International Conference on Harmonisation (ICH).

3. Common and scientific names

3.1 Common Name: Black cohosh

Note: According to the American Herbal Products Association's *Herbs of Commerce*, 2nd ed.,[1] the standard common name of *Actaea racemosa* is black cohosh. Any other species referred to as black cohosh is considered an adulterant under botanical dietary supplement GMPs. Each of the other *Actaea* species has their own common name.

3.2 Other Common Names

English: Black bugbane, snakeroot, rheumatism weed, fairy candle, tall bugbane, macrotys, macrotrys, battleweed, columbine-leaved leontice, cordate rattle top, rattleweed, false cohosh, papoose root

Chinese: *Zong zhuang sheng ma* (总状升麻)

French: Actée à grappes noires, cimicaire à grappes, chasse-punaises, cimifuge, herbe à punaise, serpentaire noire

German: Traubensilberkerze, Wanzenkraut, Frauenwurzel, langtraubiges Christophskraut, Nordamerikanische Schlangenwurzel, schwarze Schlangenwurzel

Italian: Cimicifuga, serpentaria nera, radice della squaw, actea nera

Spanish: Cohosh negro, raíz de culebra negra

3.3 Latin Binomial: *Actaea racemosa* L.

3.4 Synonyms: *Cimicifuga racemosa* (L.) Nutt.

3.5 Botanical Family: Ranunculaceae

4. Botanical Description

Actaea racemosa is native only to the eastern portion of North America, although it is propagated in numerous countries. Botanical descriptions for *A. racemosa* and its adulterant species are provided in local, national, and international floras, including the *Flora of North America* and the *Flora of China* (for Chinese species of *Actaea* and *Vernonia*). Additionally, a botanical description for *Actaea* species reported as adulterants is provided in the black cohosh monograph of the American Herbal Pharmacopoeia (AHP) and is accompanied by illustrations and images.[2] A detailed morphological analysis of black cohosh populations was published by Gardner et al.[3] Identifying and differentiating among the species requires personnel trained in botany and requires the assessment of materials whose botanical characteristic features are intact.

It is believed that Chinese material belonging to the *Actaea* or *Cimicifuga* genera other than the species identified above is sometimes sold as "black cohosh."[8] *Hagers Handbuch der Drogen und Arzneistoffe* also lists other *Actaea* species, and the roots of arnica (*Arnica montana*, Asteraceae) and the rhizomes of black hellebore (*Helleborus niger*, Ranunculaceae) as possible adulterants.[9] However, there is no evidence that adulteration with arnica or black hellebore is still an issue in the current marketplace.

Sections 5-8 of this document discuss macroscopic, microscopic, genetic, and phytochemical authentication methods for *A. racemosa*. A comparison among the various approaches is presented in Table 3 at the end of section 9.

5. Identification and Distinction using Macroanatomical Characteristics

Macroscopic identification criteria for *A. racemosa* have been published in the AHP monograph,[2] in the book chapter by Hiller,[10] and in the US Pharmacopeia (USP).[11] However, there is no information on criteria to distinguish black cohosh macroscopically from related *Actaea* species and such a distinction may be challenging. Therefore, other means for authentication should be used. The AHP monograph does have photographs of authentic *A. racemosa* and *A. podocarpa*, as well as Chinese samples. These species are morphologically distinguishable from each other.

6. Identification and Distinction using Microanatomical Characteristics

Detailed microscopic descriptions of *A. racemosa* are found in numerous references.[2,12-14] In addition, the *Botanical Microscopy Atlas*[12] contains images of transverse sections of the root and rhizome of *A. pachypoda* and *A. podocarpa*. Applequist lists the microscopic characteristics of North American species of *Actaea* growing in the same area. Whole and cut root material of *A. racemosa*, *A. pachypoda*, and *A. podocarpa* can be distinguished based on the number and shape of xylem strands in the rhizome and the root. *Actaea rubra* is less easily distinguished from *A. racemosa*, and, even if there are differences in the number and width of the vascular bundles, experience and much attention to detail is required to distinguish the two species.[14] The AHP monograph contains illustrations and photographic images of authentic *A. racemosa*, but not its adulterants. In the Chinese literature, there is information related to the microscopic differentiation of a number of *Actaea* species, including the three official species listed by the *Chinese Pharmacopoeia* for *sheng ma*, a number of other species from the same genus, and common adulterants.[15] The characteristic features for both transverse sections and powder forms are covered. Generally speaking, the differences among the Chinese *Actaea* species are subtle and it is challenging to differentiate them because they share many similar microscopic features. However, a comparison of the microscopic features of Chinese *Actaea* materials with black cohosh is lacking.

Based on the available authoritative resources, there is no single reference that contains information on *A. racemosa* and all its known current adulterants, e.g., the Chinese *Actaea* species, co-occurring Appalachian species, *Acilepis aspera*, or *Serratula chinensis*.

Comments: While microscopic distinction of *A. racemosa* and closely related North American *Actaea* species using transverse sections of whole roots has been described, it is unclear if a distinction can be achieved with powdered root material. To the best of the knowledge of the author and peer reviewers of this document, there is no detailed comparison of microanatomical characteristics among black cohosh and Chinese *Actaea* species. Therefore, the sole use of microscopy for the authentication of *A. racemosa* and for the detection of its adulterants should be considered inadequate.

7. Genetic Identification and Distinction

Methods described in the following literature were evaluated in this review: Zerega et al.[16] and Baker et al.[17]

Comments: Several scientists have developed DNA-based methods for *Actaea* species identification and detection of adulterants. In 2002, Zerega et al.[16] published a method using *Amplified Fragment Length Polymorphism* (AFLP); however, newer methods utilizing DNA sequence-based methods are superior in specificity and reproducibility. In the publication by Baker et al.,[17] DNA barcodes that amplify specific regions of the genome that are variable among *Actaea* species are used. DNA sequence-based methods utilizing "universal" (vs. species-specific) primers can also amplify unexpected adulterants and can provide an extremely reliable and robust system not only for distinguishing among closely related *Actaea* species, but also in detecting mixtures with other adulterants. In a collaboration between industry and the National Institute of Standards and Technology (NIST), *A. racemosa* leaf and root Standard Reference Materials (SRMs) with associated DNA barcodes for two validated gene regions have been developed; these DNA barcodes were validated for specificity across numerous samples of target and adulterant species and have demonstrated a 100% probability of identification.[18] The NIST SRMs with associated DNA barcodes and validation data will be made available by the end of 2015 (Catherine Rimmer e-mail communication, June 25, 2015). This authentication method has been successfully utilized across a wide range of starting materials, including dried and fresh leaves and roots in whole, cut, and powdered

Table 1. Scientific names, family, and common names of known black cohosh adulterants**

Species[a]	Synonym(s)[b]	Family	Common name[c]	Other common names[d]
Actaea cordifolia DC.	*A. rubifolia* (Kearney) Kartesz; *Cimicifuga rubifolia* Kearney; *C. racemosa* var. *cordifolia* (DC.) A. Gray	Ranunculaceae		Appalachian bugbane
Actaea pachypoda Elliott	*A. brachypetala* var. *coerulea* DC.; *A. brachypetala* var. *microcarpa* DC.; *A. pachypoda* f. *microcarpa* (DC.) Fassett	Ranunculaceae		White baneberry, doll's eyes, necklace weed
Actaea podocarpa DC.	*A. americana* Prantl; *C. americana* Michx.; *C. podocarpa* (DC.) Elliott	Ranunculaceae		Yellow cohosh, mountain bugbane
Actaea rubra (Aiton) Willd.	*A. arguta* Nutt. *A. caudata* Greene; *A. erythrocarpa* (Fisch.) Kom.; *A. rubra* subsp. *arguta* (Nutt.) Hultén; *A. viridiflora* Greene	Ranunculaceae		Red baneberry
Actaea cimicifuga L.	*C. foetida* L.	Ranunculaceae	Chinese cimicifuga	Skunk bugbane, *xi sheng ma* (西升麻)[e], *sheng ma* (升麻)[f]
Actaea dahurica (Turcz. ex Fisch. & C.A. Mey.) Franch.	*A. pterosperma* Turcz. ex Fisch. & C.A. Mey.; *Actinospora dahurica* Turcz. ex Fisch. & C.A. Mey.; *C. dahurica* (Turcz.) Maxim.	Ranunculaceae	Chinese cimicifuga	Dahurian bugbane, *bei sheng ma* (北升麻)[e], *xing an sheng ma* (兴安升麻)[f]
Actaea heracleifolia (Kom.) J.Compton	*C. heracleifolia* Kom.	Ranunculaceae	Chinese cimicifuga	Large-leaf bugbane, *guan sheng ma* (关升麻)[e], *da san ye sheng ma* (大三叶升麻)[f]
Actaea simplex (DC.) Wormsk. ex Prantl	*A. cimicifuga* var. *simplex* DC.; *C. simplex* (DC.) Wormsk. ex Turcz. ; *C. ussuriensis* Oett., *Thalictrodes simplex* (DC.) Kuntze	Ranunculaceae		*dan sui sheng ma* (单穗升麻)[f]
Acilepis aspera (Buch.-Ham.) H.Rob.	*Vernonia aspera* Buch.-Ham.; *V. roxburghii* Less.; *V. teres* Wall.; *Xipholepis aspera* (Buch.-Ham.) Steetz	Asteraceae		cao ye ban jiu ju (糙叶斑鸠菊)[f]
Serratula chinensis S. Moore	*Centaurea missionis* H. Lév.	Asteraceae		*guang dong sheng ma* (广东升麻)[e], *hua ma hua tou* (华麻花头)[f]

**Plants listed in this table include plants that have historically been known to be adulterants of black cohosh but do not necessarily reflect plants that are known to be of significant potential or actual adulteration in the herb marketplace in recent years.
[a]The Plant List and the Tropicos database.[4,5]
[b]The Plant List and the Tropicos database.[5] A comprehensive list of synonyms can be accessed through both websites.
[c]*Herbs of Commerce*, 2nd ed.[1]
[d]*Herbs of Commerce*, 2nd ed.,[1] the USDA PLANTS Database,[6] and Pengelly and Bennett.[7]
[e]Chinese common name in trade.
[f]Chinese scientific botanical name.

Note: For *Actaea/Cimicifuga*, the accepted species name of the species may differ between The Plant List and the Tropicos database; The Plant List lists *Actaea cimicifuga* L. as the accepted Latin binomial, while Tropicos lists *Cimicifuga foetida* L. In such cases, the names from The Plant List are indicated in this table as the accepted species name.

form; however, validated methods for more highly processed materials such as dried extracts are not currently available. Because genetic identification is unable to determine plant part, morphological, microscopic, or chemical evaluation is also necessary for materials in powdered form.

8. Chemical Identification and Distinction

A large number of analytical methods have been published that are used for identifying *A. racemosa* roots/rhizomes and root/rhizome extracts based on their chemistry. These methods are cited in the Laboratory Methods section below. For some methods, distinction based on the phytochemical profile may require a detailed knowledge of the constituents of black cohosh and its adulterants. The important components in *A. racemosa* and its adulterating species are listed below. When distinction is based on chromatographic or spectral patterns, identification of specific constituents may not be necessary.

8.1 Chemistry of *Actaea racemosa* and the Potential Adulterants

Actaea racemosa: Besides the prominent triterpene glycosides (Figure 1) of the 9,19-cycloartenol type, phenolic acids, tannins, fatty acids, and nitrogen-containing compounds like alkaloids, nucleobases, nucleosides, and phenolic amides and amines have been described from black cohosh.[2,7,10,19-24] Ganzera et al.[25] and Avula et al.[26,27] identified the major triterpene glycosides as actein (**1**), 23-epi-26-deoxyactein (**2**), and cimiracemoside A (**3**) (different from cimiracemoside A isolated by Shao et al.[28]). Additional triterpene glycosides occurring in large concentrations are cimicifugoside H-1 (**4**), cimigenol-3-*O*-xyloside (**5**), cimigenol-3-*O*-arabinoside (**6**), 23-*O*-acetylshengmanol-3-*O*-xyloside (**7**), and 23-*O*-acetylshengmanol-3-*O*-arabinoside (**8**).[29-33]

The phenolic acids (Figure 2) isolated from black cohosh

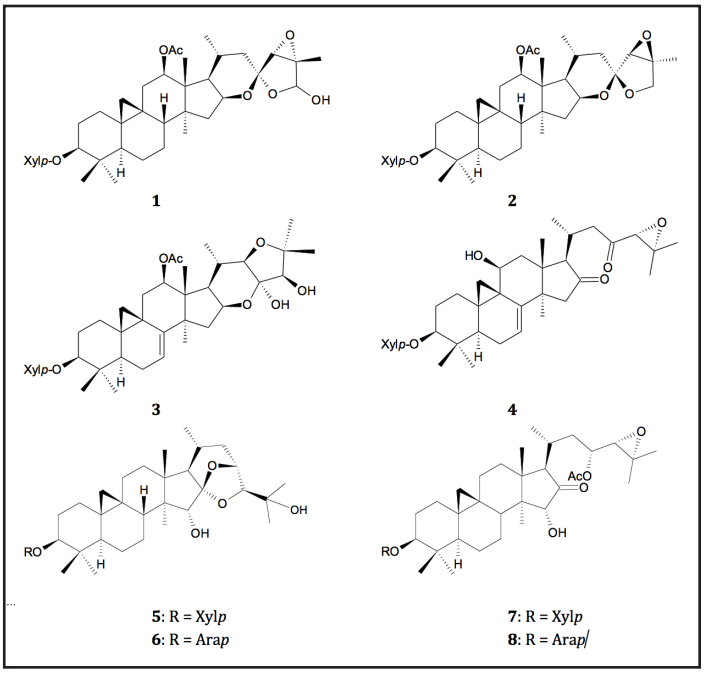

Figure 1: Major triterpene glycosides in black cohosh

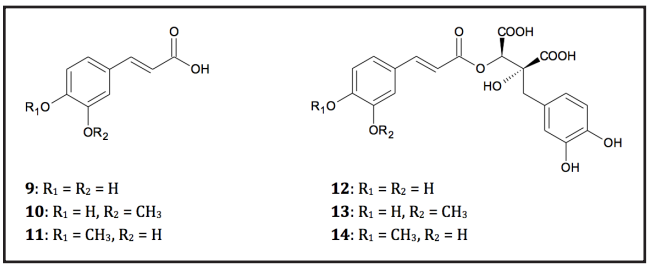

Figure 2: Phenolic acids found in black cohosh

are caffeic acid (**9**), ferulic acid (**10**), and isoferulic acid (**11**), either in simple form or as esters of fukiic acid or piscidic acid, e.g., fukinolic acid (**12**, syn: cimicifugic acid KC), cimicifugic acids A (**13**, syn: cimicifugic acid KF), B (**14**, syn: cimicifugic acid KI), E (syn: cimicifugic acid PF), and F (syn: cimicifugic acid PI).[31,34]

Research to find new phytochemicals in black cohosh roots and rhizomes has led to a rather well-known phytochemical profile for this plant, but also to a proliferation of confusing and sometimes erroneous common names, in particular for the cycloartane triterpenes. A more rational approach was described by Qiu et al.[35]; it remains to be seen if the rather lengthy names proposed in the Qiu et al. paper will be accepted by the scientific community. Examples of the new names for some of the major triterpene glycosides are given in Table 2.

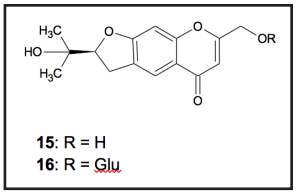

Figure 3: Structures of cimifugin (15) and cimifugin-3-*O*-glucoside (16)

Actaea cordifolia: The major phenolic acid in *A. cordifolia* is **12**. The roots/rhizomes also contains **13** and **14**. The authors were unable to determine the identity of the predominant triterpene glycosides, but detected smaller amounts of **1**, **2**, **4**, and 25-*O*-acetylcimigenol-3-*O*-xyloside. In addition, the analysis revealed the occurrence of the dihydrofurochromone cimifugin (**15**) and cimifugin-3-*O*-glucoside (**16**) (Figure 3).[36] The analysis of *A. cordifolia* by a different group led to the identification of **6** as a major triterpene glycoside, but **1**, **2**, and **4** were not found.[37] Based on the contradictory results, additional work needs to be done to conclusively establish the triterpene glycoside composition of *A. cordifolia*.

Actaea pachypoda: The predominant phenolic acid is **12**, with lesser amounts of **13** and **14**.[36] Triterpene glycoside fingerprints of *A. pachypoda* have been established by HPLC-MS, and identified **1-3** and **5-7**, although only **1** and **2** were consistently found.[26,30,36]

Actaea podocarpa: Root/rhizome material from *A. podocarpa* was found to contain mainly **10**, 2-feruloylpiscidic acid, and **13**, and only traces of **12** and **14**.[36,37] The analysis of the triterpene glycosides showed the presence of **5** and **6**. Importantly, **1** was absent and **2** was found in only one of the four samples analyzed.[26,30,36,37] A number of unique triterpene glycosides, the podocarpasides, were reported by Ali et al.[38,39]

Actaea rubra: A phytochemical fingerprint study by

Table 2. Nomenclature of major triterpene glycosides from *A. racemosa* according to Qiu et al.[35]

Original name	Synonyms	New name
23-*O*-acetylshengmanol-3-*O*-xyloside (**7**)		(23*R*)-23-acetoxy-(24*S*)-24,25-epoxy-(15*R*)-15-hydroxy-16-oxo-3-*O*-β-D-xylopyranosylactanoside
Actein (**1**)	Shengmating	(12*R*)-12-acetoxy-(24*R*,25*S*)-24,25-epoxy-(26*R*&*S*)-26-hydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside
Cimigenol-3-*O*-arabinoside (**6**)	Cimiracemoside C, cimicifugoside M	(15*R*)-15,25-dihydroxy-3-*O*-α-L-arabinopyranosylacta-(16*S*,23*R*,24*S*)-16,23;16,24-binoxoside
Cimigenol-3-*O*-xyloside (**5**)	Cimicifugol-xyloside, cimigenoside, cimicifugoside	(15*R*)-15,25-dihydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*,24*S*)-16,23;16,24-binoxoside
Cimiracemoside A (**3**)	Cimiracemoside F	(12*R*)-12-acetoxy-7,8-didehydro-(23*R*,24*R*)-23,24-dihydroxy-3-*O*-β-D-xylopyranosylacta-(16*S*,22*R*)-16,23;22,25-binoxoside
23-Epi-26-deoxyactein (**2**)	26-Deoxyactein, 27-deoxyactein	(12*R*)-12-acetoxy-(24*R*,25*R*)-24,25-epoxy-3-*O*-β-D-xylopyranosylacta-(16*S*,23*R*)-16,23;23,26-binoxoside

Jiang et al.[36] found **12-14** as major phenolic acids in *A. rubra*. Depending on the material, the triterpene glycosides identified were **1**, **2**, and **5-7**, but **3** was notably absent in all the samples analyzed.[26,30,36,40] In addition, a number of cimigenol derivatives and rubraside A have been isolated from the roots.[40] According to one study, the compositions of *A. rubra* and *A. pachypoda* are similar and cannot be distinguished by chemical means.[30]

Actaea cimicifuga: A large number of new triterpene glycosides have been reported from the roots and rhizomes of *A. cimicifuga*, but quantitative measurements of them are lacking. Only one paper presented a quantitative analysis of the contents of the major triterpene glycosides **2**, **4**, **5**, and 25-*O*-acetylcimigenol-3-*O*-xyloside.[41] The same four triterpene glycosides have also been reported by He et al,[37] but **5** was absent in the material analyzed by Wang et al.[42] The main phenolic acids are **12** and **13**, according to Jiang et al.[36] The presence of **15** and **16**, and the triterpene alkaloid cimicifugadine (syn: cimicifine A), can be used to distinguish *A. cimicifuga* material from black cohosh.[23,34,37,42]

Actaea dahurica: Tang and Eisenbrand[43] described **5**, **7**, cimigenol, dahurinol, shengmanol-3-*O*-xyloside, and 24-*O*-acetylanhydroshengmanol-3-*O*-xyloside from *A. dahurica* rhizomes. The presence of **2**, **5**, and **7** was reported by other research groups.[36,37] The main phenolic acids are **11**, **12**, and **14**.[36] As with *A. cimicifuga*, the presence of **15**, **16**, and cimicifugadine can be used to distinguish *A. dahurica* material from black cohosh.[23,36,37,42]

Actaea heracleifolia: Seven known triterpene glycosides – **5**, **7**, **8**, 24-*epi*-24-*O*-acetylhydroshengmanol-3-*O*-xyloside, cimiaceroside B, 25-*O*-acetylcimigenol-3-*O*-β-D-xyloside, and 25-*O*-anhydrocimigenol-3-*O*-xyloside – were isolated from the rhizomes of *A. heracleifolia*.[44] Importantly, both **1** and **2** reportedly do not occur in the species.[23,36,37,42] The phenolic acids **12-14** have been described in *A. heracleifolia* material by Jiang et al.[36] at low levels, but not by He et al.,[37] who detected 2-feruloylpiscidic acid and 2-isoferuloylpiscidic acid instead. Since **15**, **16**, and cimicifugadine are found in *A. heracleifolia* roots and rhizomes, these compounds can be used to distinguish it from black cohosh.[23,36,42]

Actaea simplex: Cycloartenol triterpene glycosides are also predominant in the roots/rhizomes of *A. simplex*. Six new triterpenes have been isolated by Kuang et al.[45,46] The occurrence of **1**, **2**, **4**, and **5** in the roots/rhizomes remains controversial,[36,37,42] but if confirmed, these compounds are present most likely at very low levels. The phenolic acids **12-14** have been reported from the roots and the rhizomes.[36,37] The presence of **15** and **16**, which have been reported from *A. simplex*, is indicative of adulteration of black cohosh with Chinese *Actaea* species. However, the identification of adulteration with *A. simplex* should be based on a comparison of the overall chemical fingerprint with authentic material.[36,37]

Acilepis aspera: There are no published reports available in the chemical and other scientific literature on the chemical composition of *Acilepis aspera* roots.

Serratula chinensis: The roots of *S. chinensis* were investigated by Ling et al., who isolated seven ecdysteroids and five cembrosides, although only three of them were subsequently identified.[47,48] None of the 9,19-cycloartenol type triterpene glycosides or the phenolic acids typical for *Actaea* species have been reported from this plant.

8.2 Laboratory Methods

Note: Unless otherwise noted, all methods summarized below are based on using only the sub-aerial roots and rhizomes of authentic black cohosh and its adulterants.

8.2.1 HPTLC

Methods from the following sources were evaluated in this review: Upton,[2] the USP 34,[11] the EP 7.5,[13] Gafner et al.,[30] Wagner and Bladt,[49] Zheng et al.,[50] Ankli et al.,[51] and Verbitski et al.[52]

Comments: The HPTLC analysis of *Actaea* is a rare instance where the majority of authors have relied on the same stationary and mobile phases. The thorough validation, proven ability to detect adulteration, and flexibility

to target various phytochemicals depending on the detection approach make the ethyl formate–toluene–formic acid (3:5:2, v/v) mobile phase using HPTLC silica gel 60 F_{254} plates the method of choice for HPTLC analysis of black cohosh (Figure 4).[2,11,13,30,51-53]

Since method validations were conducted using the sample preparation and detection system described in references 11 and 51, the consensus of authors and expert peer reviewers of this Laboratory Guidance Document is that this procedure is the most suitable in a routine QC lab. While this method is capable of distinguishing various *Actaea* species based on the chemical fingerprint, the detection of adulterating species – in particular when such species are added to *A. racemosa* – remains challenging. Some of the related North American species exhibit a constituent profile similar to black cohosh; in addition, the constituent profile may vary depending on the geographic location and manufacturing process, although according to Eike Reich of CAMAG, the chemical composition of black cohosh is rather consistent (Eike Reich e-mail communication, November 19, 2014)

To detect adulteration with Chinese *Actaea* species, the presence of **15** (found in, e.g., *A. cimicifuga*, *A. dahurica*, *A. heracleifolia*, and *A. simplex*) can be verified using boric acid-oxalic acid reagent reported by Ankli et al.[51] The application of boric acid-oxalic acid reagent leads to a strong fluorescence of **15** under UV light at 366 nm, and allows the detection of as little as 1% of *A. cimicifuga* and *A. simplex* in black cohosh (Figure 5). For obvious reasons, the boric acid-oxalic acid reagent does not allow the detection of adulteration with *Actaea* species where **15** is absent (e.g., *A. pachypoda*, *A. podocarpa*, and *A. rubra*). Detection of admixture of *A. heracleifolia* or *A. cimicifuga* at levels below 5% has been achieved using the antimony chloride detection reagent.[51]

8.2.2 HPLC and UHPLC

Methods described in the following literature were evaluated in this review: Upton,[2] the USP 34,[11] the EP 7.5,[13] Ma et al.,[23] Ganzera et al.,[25] Avula et al.,[26,27] Li et al.,[29] Gafner et al.,[30] Jiang et al.,[31,36,54] He et al.,[37,55] Wang et al.,[42] and Looney.[56] Specific comments on strengths and weaknesses of each of the methods are listed in Appendix 1, Table 4.

Comments: The authentication and detection of black cohosh adulteration by HPLC or UHPLC should be based on a chemical fingerprint, and the incoming raw material compared to fingerprints from a number of representative authenticated samples by statistical means. For routine quality control, a quick and easy sample preparation method is provided in the *European Pharmacopoeia*.[13] The solvent of choice in most cases is a mixture of MeOH-water (between 75 and 100% methanol, v/v) or EtOH-water (1:1 or 7:3, v/v).

Based on the run time, quality of separation, and extensive validation, the HPLC-ELSD method presented in references 2 and 37 is a good choice, but system suitability parameters have to be developed. Such parameters have been specified only by the compendial methods outlined in

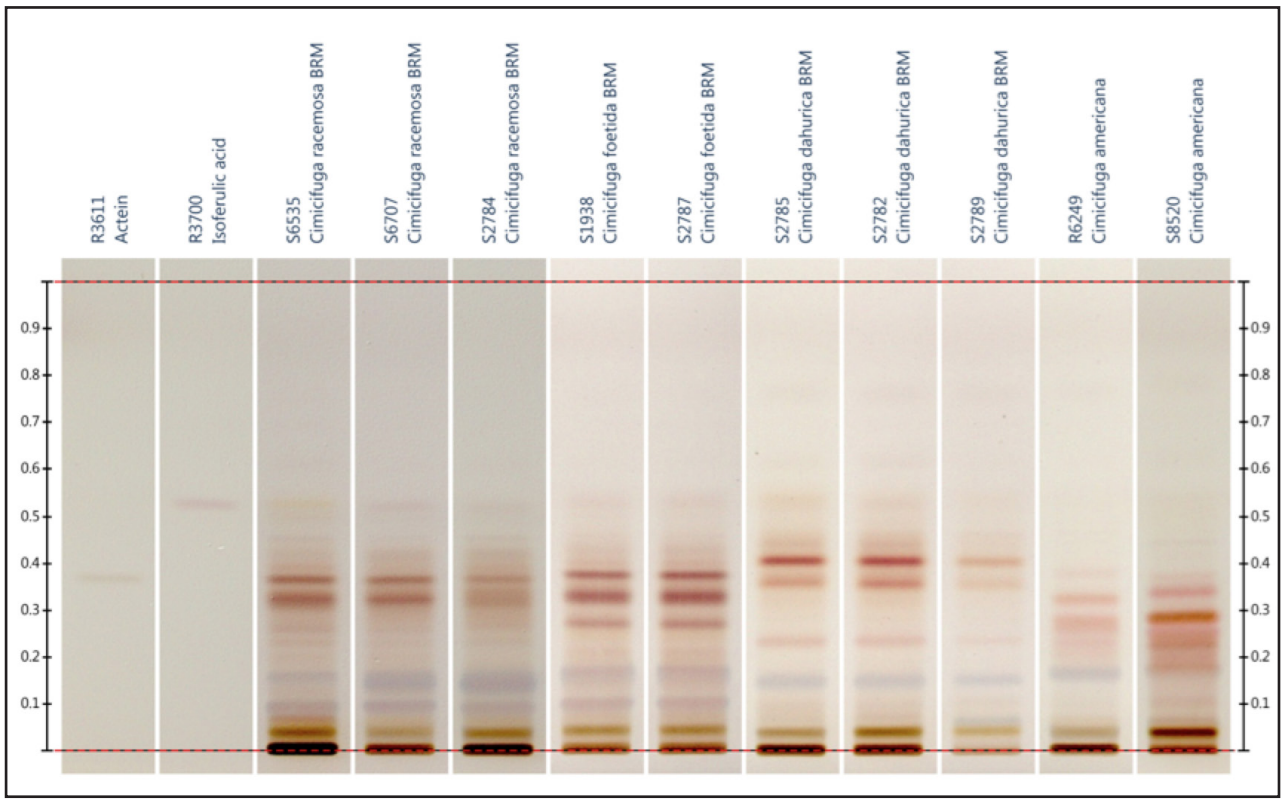

Figure 4: HPTLC analysis of root extracts of black cohosh, *Actaea podocarpa* (syn. *Cimicifuga americana*), and two Chinese *Actaea* spp., *A. dahurica* (syn. *C. dahurica*) and *A. cimicifuga* (syn. *C. foetida*).[53] Detection: visible light after derivatization with sulfuric acid reagent. Image provided by CAMAG (Muttenz, Switzerland).

the *United States Pharmacopeia*[11] and the *European Pharmacopoeia*.[13] If the run time is of essence, the conditions described by Avula et al.[27] are by far the quickest, but require having a UHPLC instrument that can run under higher pressure. However, no system suitability parameters are provided for the method.

8.2.3 MS-Fingerprinting

Methods described in the following literature were evaluated in this review: Huang et al.[57] and Harnly et al.[18]

Comments: Flow-injection mass spectrometry (FIMS), in which the ion spectrum is summed over the injection interval, provides complex spectral fingerprints that, like those for HPLC or NMR, can be used to compare unknown materials to a series of authentic materials using statistical means. The sample preparation is identical to that used for HPLC. Electrospray ionization was used by Huang et al.[57] and Harnly et al.[18] but the use of other ionization sources is possible as well.

Normal mass calibration is required for alignment of the spectral fingerprints. The authors used chemometric analysis, e.g., principal component analysis (PCA), for visual inspection of the data and soft independent modeling of class analogy (SIMCA) to provide statistical evaluation of the degree of discrimination. As with other chemically based methods, this approach works well with ingredients that are consistently processed in the same way. This statistics-based authentication is state of the art for dietary supplements. The analysis is very short and environmentally friendly due to low solvent use. Data on method validation are not available. Initial costs for the instrumentation are high.

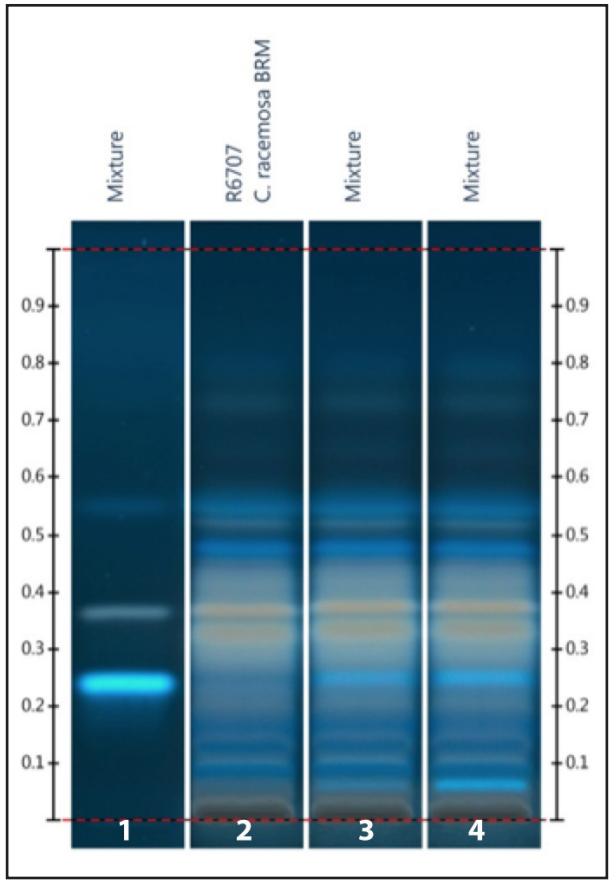

Figure 5: HPTLC evaluation of black cohosh adulteration with *Actaea cimicifuga* and *A. simplex*. Detection: UV light (366 nm) after derivatization with boric acid/oxalic acid reagent.[53] Lane 1: cimifugin, actein, isoferulic acid (with increasing Rf value); lane 2: *A. racemosa*; lane 3: 5% *A. cimicifuga* in *A. racemosa*; lane 4: 5% *A. simplex* in *A. racemosa*. Image provided by CAMAG (Muttenz, Switzerland).

8.2.4 NMR

One NMR-based authentication method was evaluated in this review (Harnly et al.[18]).

Comments: Whole, cut, or powdered samples are extracted using aqueous methanol followed by drying and re-solvation in DMSO-d_6. For extracts, the material is directly dissolved in DMSO-d_6. Harnly et al.[18] use one-dimensional ^{1}H-NMR to establish spectral fingerprints of the crude extracts. The fingerprints allow differentiating *A. racemosa* from other *Actaea* species using statistical evaluation, e.g., PCA and SIMCA, as well as identification and quantification of some of the metabolites present in the samples.

The method provides state-of-the-art statistics-based authentication. The results show that the NMR approach is able to clearly distinguish *A. racemosa* from other *Actaea* samples. As with other statistics-based evaluations, added materials (e.g., carriers, processing aids) or variations in the manufacturing process will modify the outcome of the PCA and thus may cluster the material outside the acceptable range. Therefore, the construction of a library containing authenticated materials of the same composition as the analyte is necessary. Expert analysts are required to set up the right parameters and run the instrument. The analysis time is short and ecologically responsible due to the low amount of solvent used. As a result of the reproducibility using NMR, new samples can be directly compared to samples run earlier without having to rerun the whole series. System suitability for any botanical analysis is the same: the ^{1}H line shape and the ^{1}H sensitivity have to comply with the probe specifications. In addition, the temperature must be stable to 0.1°C. However, the sample preparation for whole, cut, or powdered raw material is time-consuming due to initial extraction time and the need to freeze-dry the extract before analysis in order to avoid a large signal from residual water. Data on method validation are not available. Initial costs for the instrumentation are high.

9. Conclusion

Authentication of cut or powdered black cohosh rhizome is challenging due to the existence of closely related and sometimes co-habiting *Actaea* species with similar morphological features and chemical composition. On rare occasions, phenotypes have displayed far greater variation than the genotypes.[18] The need for sound analytical methods is further emphasized by the abundance of materials from China sold as "black cohosh" but composed of root and sub-aerial material from entirely different species. For authentication of raw material, a combination of a physi-

cal assessment test (ideally using the whole plant) and/or a genetic approach[17,18] combined with chemical identification methods is needed. For materials where DNA-based technologies are applicable, they have given the most accurate results. Rare cases of misidentification of *A. racemosa*, *A. pachypoda*, and *A. podocarpa* rhizome based on chromatographic authentication methods have been reported by Hartwig Sievers (e-mail communication, September 29, 2014) and Harnly et al.[18]

Authentication or detection of adulteration in extracts remains difficult since there is currently no method available for a chemical compound or phytochemical class that is characteristic for *A. racemosa*. (Some of the recently discovered alkaloids may be useful for species authentication, but no methods have been published to date.) The presence of **3** and absence of **15** and **16** are indicators for authentic black cohosh, but relying on the presence/absence of a few marker compounds for the authentication of black cohosh is insufficient. Any method for chemical authentication must be based on a fingerprint, which means the entire spectrum of chemical compounds present has to be evaluated using appropriate software for statistical analysis. Several published HPTLC methods have shown their ability to distinguish black cohosh and its major adulterants.[2,11,13,30,51-53] The method of choice for detection of adulterants added to authentic *A. racemosa* material by HPTLC is described by Ankli et al.[51] Most of the published HPLC methods will be able to authenticate black cohosh, but proving that it is only black cohosh (without any admixture of other material) is a difficult task. When using HPLC, the sample preparation outlined in EP 7.5[13] combined with the chromatographic method reported in references 2 and 37 is a good choice. Despite its main purpose for quantitative assessment, the HPLC-ELSD method in the *European Pharmacopoeia*[13] has also been successfully applied to detect adulteration of black cohosh and can be used for additional confirmation (Pilar Pais personal communication, May 30, 2014). For increased specificity, the use of an MS detector in addition to the ELSD should be considered.

Recent publications using direct analysis by FIMS[18,57] and NMR[18] with subsequent chemometric evaluation to distinguish between *A. racemosa* and other *Actaea* species offer a unique approach in botanical authentication. These methods combine simple sample preparation and rapid analysis. NMR offers unprecedented signal stability (the intensity of the NMR signals change very little over time, therefore allowing the comparison of spectra of new materials with archived data, effectively eliminating the need to acquire data for standard materials before each NMR experiment). MS and ^{1}H-NMR have the proven ability to fully characterize black cohosh and its adulterants. The results show that FIMS and NMR (see below) perform equally well in distinguishing the various *Actaea* species; however, the results also show the limitations of chemistry-based identification methods. Despite a robust statistical evaluation of the results, one sample of *A. pachypoda* root clustered within the black cohosh samples, and one sample of *A. racemosa* root fell outside the 95% confidence interval set for authentication of black cohosh. Both samples were correctly identified using a DNA barcoding approach.[18] These cases of misidentification may be rare, and have been related to the unusual chemical composition of the materials.

Note: A number of identity tests for black cohosh materials are offered by third-party analytical laboratories. According to input from six contract laboratories, the testing methods include microscopy, DNA barcoding, HPTLC, and HPLC-UV. Additional testing methods (HPLC-MS or near-infrared [NIR] methods) can be developed upon request.

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Table 3. Comparison among the different approaches to authenticate *A. racemosa*.

Method	Applicable to	Pro	Contra
Macroscopic	- Unprocessed plant parts	- Quick - Inexpensive	- No automation/statistics - Outcome relies on analysts' expertise - Challenge for c/s material
Microscopic	- Unprocessed plant parts	- Quick - Inexpensive	- No automation/statistics - Outcome relies on analysts' expertise - Challenge to distinguish closely related species
Genetic	- Unprocessed plant parts - Cut and sifted - Powdered - Many liquid extracts	- Able to distinguish closely related species - Reliable	- Labor-intensive sample preparation and analysis - Expensive equipment - Unable to differentiate plant parts
HPTLC	- Cut and sifted - Powdered - Extracts	- Quick - Affordable equipment - Adulteration with 5% *Actaea cimicifuga*, *A. heracleifolia*, *A. dahurica*, and 10% *A. podocarpa* can be detected[13] - No statistics required	- No statistics - Detection of adulteration often challenging[b] - Need for reference standard compounds
HPLC-UV	- Cut and sifted - Powdered - Extracts	- Standard equipment in many laboratories - Ideal for compounds with strong chromophore (e.g., phenolic acids) - Adulteration with cimifugin-containing *Actaea* species can be detected	- Expensive equipment - Mostly quantitative (less specific than HPLC-UV/MS) - Low sensitivity for triterpene glycosides - Unable to distinguish overlapping peaks - Detection of adulteration often challenging[b] - Need for reference standard compounds - Not suitable to obtain a fingerprint of triterpene glycosides
HPLC-ELSD	- Cut and sifted - Powdered - Extracts	- Suitable for fingerprinting of triterpene glycosides	- Expensive equipment - Unable to distinguish overlapping peaks - Detection of adulteration often challenging[b] - Need for reference standard compounds - Unable to distinguish co-eluting compounds (e.g., when compared to MS detector)
HPLC-UV/MS	- Cut and sifted - Powdered - Extracts	- Qualitative and quantitative - High sensitivity - State-of-the-art statistical evaluation possible[23] - Adulteration with cimifugin-containing *Actaea* species can be detected	- Expensive equipment - Detection of adulteration often challenging[b] - Need for reference standard compounds
Standalone MS (FIMS)	- Cut and sifted - Powdered - Extracts	- High sensitivity - Discriminates among *Actaea* species - State-of-the-art statistical evaluation possible - Identification of important ions	- Expensive equipment[c] - Complex initial setup of parameters - Quality of data depends on ability to ionize analyte of interest
^{1}H-NMR	- Cut and sifted - Powdered - Extracts	- Long-term reproducibility - Discriminates among *Actaea* species - State-of-the-art statistical evaluation possible - Identification/quantification of specific metabolites	- Equipment expensive but becoming more common in labs[c] - Need for spectral database libraries - Needs at least 4' x 7' floor space
HMBC-NMR	- Cut and sifted - Powdered - Extracts	- Long-term reproducibility - Suitable for fingerprinting of triterpene glycosides - Discriminates among *Actaea* species	- Equipment expensive but becoming more common in labs[c] - Need for spectral database libraries - Needs at least 4' x 7' floor space

[a]Only whole and cut and sifted (c/s) materials.
[b]In particular, detection of adulteration in mixtures of *A. racemosa* and related *Actaea* species, due to variability in triterpene-glycoside composition within *A. racemosa* populations and due to occurrence of same compounds in other *Actaea* species.
[c]Costs for high-resolution mass spectrometers and NMR instruments are generally above US $250,000. A low-cost 300 MHz NMR for natural products analysis can be obtained for US $150,000.

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Appendix 1

Table 4: Comments on the published HPLC methods for *A. racemosa*.

Reference	Comments
Upton,[2] He[37]	This is a validated HPLC-ELSD method with an acceptable duration of run and good peak shapes. The fingerprint data show an *A. racemosa* triterpene-glycoside pattern that is distinct from 2 North American and 7 Asian *Actaea* species. The sample preparation is lengthy based on the 24-hr extraction period, but consists only of a few handling steps. In order to extend column life, a mobile phase containing 0.1% formic acid is preferred. There is no information on peak identity using the validated ELSD detection and not all peaks are well separated.
USP,[11] Li,[29] Gafner[30]	This HPLC-ELSD method has good peak shapes and a reasonable separation. It has been adopted as official method by the USP. The USP monograph contains detailed parameters for system suitability. However, the sample preparation is labor-intensive and the HPLC run time of 70 min is unnecessarily long since no peaks elute after 55 min. Using the validated ELSD detection will not provide information on peak identity. Not all peaks are well separated.
EP[13]	This HPLC-ELSD method has been validated and contains detailed parameters for system suitability. The sample preparation is quick and easy. The run time is reasonably short. The method was developed to quantify triterpene glycosides and there is no published data on its ability to authenticate black cohosh (the EP 7.5 standard method for authentication relies on HPTLC). Using the validated ELSD detection will not provide information on peak identity.
Ma[23]	This HPLC-MS method has been tested on 9 *A. racemosa* populations and 15 additional *Actaea* species. The statistical evaluation of HPLC-TOFMS fingerprints using PCA is state of the art. The results show that authentication of *A. racemosa* is possible based on the presence of **6** combined with the absence of **15** and **16**. Sample preparation is time-consuming and labor-intensive. An HPLC system that can handle ternary solvent systems is required. The separation is reasonable, but the run time of 103 min is quite long. The method has not been validated.
Ganzera[25]	This HPLC-ELSD method has a quick and simple sample preparation procedure. The run time is reasonably short but the chromatogram shows some overlapping peaks. The method has proven its ability to differentiate *A. racemosa* from *A. dahurica* and *A. cimicifuga*, but the data are limited to one sample per species. The composition of the mobile phase requires an HPLC system that can handle ternary solvent systems. The use of an ELSD detector will not provide information on peak identity. The method has not been validated.
Avula[26]	This is another HPLC-ELSD approach with a quick sample preparation method. The conditions lead to a good separation for **1**, **2**, and **3**, but the chromatogram shows some overlapping of later eluting compounds, and the run time is long (75 min). The method has been validated (according to the authors) for authentication by testing 4 populations of *A. racemosa* and 3 North American *Actaea* species, but not in terms of quantitative analysis. The composition of the mobile phase requires an HPLC system that can handle ternary solvent systems. The use of an ELSD detector will not provide information on peak identity.
Avula[27]	This is a validated method combining UHPLC with three detectors (UV/Vis, ELSD, and MS). The chromatogram shows good peak shapes and separation for **1**, **2**, and **3** (the chromatograms look simple compared to those in reference 23 or 29). The run time is short and the sample preparation method is quick. The method has been shown to distinguish *A. racemosa* and 3 related North American *Actaea* species.
Jiang,[31] Jiang,[54] He[55]	The HPLC-UV (detection at 203 nm) method has been validated,[31] despite some unresolved peaks. It has proven the ability to identify adulteration if HPLC-MS (triterpene glycosides) and HPLC-UV methods (phenolic acids) are used in combination. However, the sample preparation technique using chloroform is labor-intensive and the HPLC run time lengthy (65 min).
Jiang[36]	The HPLC-UV (detection at 203 nm) method has been validated, despite some unresolved peaks. The duration of the HPLC run is acceptable. The method is capable of identifying adulteration based on the fingerprint analysis, presence of **3**, and absence of **15** (however, *A. pachypoda* also contains **3** and lacks **15**). The sample preparation technique using chloroform is labor-intensive.
Wang,[42] fingerprint	The authors present an HPLC-MS fingerprinting method that is able to distinguish between *A. racemosa* and 6 Asian *Actaea* species. The analysis of 6 commercial samples with rather different triterpene-glycoside fingerprints shows the challenges of correct authentication. The chromatographic system leads to good peak shapes and a reasonable separation, but has a long run time of 93 min. The sample preparation is labor-intensive and uses chloroform. The injection volume is high (50 µL of sample in methanol) for initial conditions consisting of MeCN-10 mM ammonium acetate in water (5:95, v/v). Crucial information on MS parameters is lacking and the method has not been validated.
Looney[56]	The author analyzed a large number (20) of *A. racemosa* populations with this HPLC-ELSD method. The sample preparation is easy but long. The run time and separation are reasonable, although there are some overlapping peaks. The use of an ELSD detector will not provide information on peak identity. The method has not proven its ability to detect adulteration with other species and has not been completely validated.

Note: Due to the presence of many triterpene glycosides with similar polarity, none of the published HPLC-UV or HPLC-ELSD methods are able to achieve baseline separation of these compounds. The term "validated" is used when a method has been validated for quantitative analysis, but not in terms of qualitative identification according to LaBudde and Harnly.[58]