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Feruloyl dopamine-*O*-hexosides are efficient marker compounds as orthogonal validation for authentication of Black Cohosh (*Actaea racemosa*) – an UHPLC-HRAM-MS Chemometrics Study

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Abstract

Due to the complexity and variation of the chemical constituents in authentic black cohosh (*Actaea racemosa*) and its potential adulterant species, an accurate and feasible method for black cohosh authentication is not easy. A high resolution accurate mass (HRAM) LC-MS fingerprinting method combined with chemometric approach was employed to discover new marker compounds. Seven hydroxycinnamic acid amide (HCAA) glycosides are proposed as potential marker compounds for differentiation of black cohosh from related species, including two Asian species (*A. foetida*, *A. dahurica*) and two American species (*A. pachypoda*, *A. podocarpa*). These markers were putatively identified by comparing their mass spectral fragmentation behavior with those of their authentic aglycone compounds and phytochemistry reports. Two isomers of feruloyl methyl dopamine 4-*O*-hexoside ($[M+H]^+$ 506) and one feruloyl tyramine 4-*O*-hexoside ($[M+H]^+$ 476) contributed significantly to the separation of Asian species in principle component analysis (PCA) score plot. The efficacy of the models built on four reasonable combinations of these markers in differentiating black cohosh and its adulterants were evaluated and validated by partial least-square discriminant analysis (PLS-DA). Two models based on these reduced dataset achieved 100% accuracy based on the current sample collection, including the model that used only three feruloyl dopamine-*O*-hexoside isomers ($[M+H]^+$ 492) and one feruloyl dopamine-*O*-dihexoside ($[M+H\text{-hexosyl}]^+$ at m/z 492).

Graphical Abstract

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Notes

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Keywords

Hydroxycinnamic Acid Amide glycoside; Hydroxycinnamic Acid ester; UHPLC-HRMS; nitrogen-containing; PCA; PLS-DA

INTRODUCTION

Black Cohosh is officially defined as the dried rhizome and roots of *Actaea racemosa* L. [formerly *Cimicifuga racemosa* (L.) Nutt.] of the family Ranunculaceae harvested in the summer [1]. It is a plant native to eastern North America with a long history of medicinal use for the treatment of menopausal symptoms in North America and Europe. In recent years, black cohosh has enjoyed a rapid rise in popular use as a non-estrogenic alternative to hormone replacement therapy, which has been shown to cause increased risk of breast cancer and cardiovascular diseases [2–4], for the amelioration of menopausal symptoms.

The majority of commercial black cohosh is still wild crafted in the United States, and overharvesting is beginning to threaten its existence in the wild [5]. As a result of increasing commercial demand and shrinking natural stocks, black cohosh products in market may be adulterated with widely cultivated and less expensive Asian *Actaea* species [6]. Asian species of *Actaea* were generally used as traditional medicines for centuries. *A. heracleifolia* (Kom.), *A. dahurica*, and *A. foetida* are listed in the Chinese Pharmacopoeia as Sheng-Ma for their anti-inflammatory, analgesic, and antipyretic properties [7]. Although these Sheng-Ma species share some constituents with black cohosh, their medical usage are different. Additionally, *A. racemosa* is often unintentionally mixed with other American *Actaea* species, such as *A. podocarpa* (yellow cohosh) and *A. pachypoda* (white cohosh) during harvest, due to its similar appearance and growing locales. These potential contaminants exerted unknown effect on menopausal symptoms [8]. Adulteration and plant misidentification of black cohosh remains a serious health concern.

Despite increasing consumption and extensive investigation, the mechanisms of its alleviation of climacteric symptoms remain largely undefined [9–11]. The phytochemical constitution of black cohosh is complex and the active ingredients remain unknown. The phytochemical investigation of black cohosh in the past several decades has focused almost exclusively on two abundant classes of compounds: triterpene glycosides and phenolic acid derivatives [12]. Cycloartane triterpenes constitute the majority of *Actaea* triterpenes, which are typically glycosylated at the C-3 position with D-xylose or L-arabinose. Abundant triterpenes such as actein and 23-*epi*-26-deoxyactein are often used as markers for the standardization of black cohosh preparations [13]. But these major constituents were also present in other American and Asian *Actaea* species [6, 14].

The phenolic compounds from *Actaea* species mainly consist of esters between the carboxyl group of caffeic, ferulic or isoferulic acid and the hydroxyl group of fukiic or piscidic acid (i.e. hydroxycinnamic acid esters). Profile analysis of phenolics and triterpenes using HPLC-UV, HPLC-MS and HPLC-ELSD (evaporative light scattering detection) have been successfully applied to distinguish *A. racemosa* from other American (i.e. *A. pachypoda*, *A. podocarpa*, and *A. rubra*), and Asian *Actaea* species [11, 14–17]. These profiling methods relied on the identification and comparison of chromatographic patterns of the complex components between *Actaea* species, which are not easy to perform routinely. Advanced expertise in both phytochemistry and MS is a prerequisite to recognizing specific marker compounds from the candidate pool which is composed of complicated and similar structural analogs.

He and co-workers indicated that two chromones, cimifugin and cimifugin glucoside, were present in Asian *Actaea* species (i.e. *A. foetida*, *A. dahurica*, and *A. simplex*), but absent in *A. racemosa* and some American *Actaea* [6, 18]. Cimifugin and cimifugin glucoside have been used as good indicators of commonly encountered Asian adulterants [8]. But cimifugin was not found in any of the *A. rubra*, *A. pachypoda*, and *A. podocarpa* species [15]. Hence, differentiation of black cohosh from its American adulterants is still problematic.

The previous work of ours [19, 20] and other researchers [6, 8, 11, 14, 16, 18, 21–23] on black cohosh components focused primarily on: 1) using ESI-negative mode to detect phenolic acids and phenolic esters; 2) using ESI/APCI-positive mode to detect triterpenes. In order to explore useful information that may be neglected in the MS positive data, principle component analysis (PCA) was performed on positive data (especially compounds eluted ahead of triterpenes on reverse phase column) in this study. Seven hydroxycinnamic acid amide (HCAA) glycosides were identified as potential markers to distinguish black cohosh and other *Actaea* species. Partial least-squares discriminant analysis (PLS-DA) models based on these markers were investigated using leave-one-out cross-validation. It was discovered that feruloyl dopamine-*O*-hexosides can be used as effective marker compounds for authentication of Black Cohosh.

EXPERIMENTAL

Materials and Chemicals

The certified rhizome/roots materials from various species of *Actaea* were purchased from the American Herbal Pharmacopoeia (AHP). The Standard Reference Material® *Actaea racemosa* was obtained from the National Institutes of Standards and Technology (NIST). The *Actaea dahurica* samples were purchased from the Internet and local stores in China. Commercial black cohosh supplements from different manufactures were purchased locally or from the Internet. All plant materials and finished products were authenticated using validated DNA sequencing authentication methods for *Actaea* [20, 24] at AuthenTechnologies LLC. These samples are all listed in Table 1. The reference compounds *trans-N*-caffeoyltyramine, *trans-N*-*p*-coumaroyltyramine, and *trans-N*-feruloyltyramine were kindly provided by Dr. Min Yang (National Engineering Laboratory for TCM Standardization Technology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences). HPLC grade methanol, acetonitrile, and formic acid were purchased from VWR International, Inc. (Clarksburg, MD, USA). HPLC water was prepared from distilled water using a Milli-Q system (Millipore Laboratories, Bedford, MA, USA).

Sample Preparation

Samples were ground into fine powder and then passed through a 20 mesh sieve. The extraction procedure was according to our previous report [25] with slight modification. Briefly, a 10 mg aliquot of each sample were extracted with 5.0 mL of methanol-water (7:3, v/v) using sonication and centrifugation as described. Then the supernatant was diluted 1:10 (v/v) with water and filtered through a 17 mm (0.20 µm) PVDF syringe filter (VWR Scientific, Seattle, WA) and stored at -80 °C until analysis. Each sample was extracted in duplicate. The injection volume for all samples was 2 µL.

UPLC-HRMSⁿ Conditions

The UHPLC-HRMSⁿ system consists of a LTQ Orbitrap XL MS with an Accela 1250 binary Pump, a PAL HTC Accela TMO autosampler, an Accela PDA detector (Thermo Fisher Scientific, San Jose, CA), and an Agilent G1316A column compartment (Agilent, Santa Clara, CA). The operating conditions of the electrospray (ESI) source were similar to our previous work [25] with the differences were as follows: sheath gas at 70 (arbitrary units), spray voltage at 4.5 kV; capillary temperature, 250 °C; capillary voltage, 50 V; tube lens offset, 120 V. MSⁿ activation parameters used an isolation width of 2.0 amu, max ion injection time of 200 ms, and normalized collision energy at 35%. The UHPLC separation was carried out on a Hypersil Gold C₁₈ column (200 mm × 2.1 mm, 1.9 µm) (Thermo Fisher Scientific, San Jose, CA) with a flow rate of 0.3 mL/min. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A gradient elution program was employed as follows: from 5% to 25% B over 0–30.0 min, to 30% B at 40.0min, to 85% B at 60.0 min, to 95 % B at 65.0 min, and then a return to the initial conditions. The column temperature was set at 50 °C.

Data Processing

Data were initially acquired as RAW files which were first converted into mzXML by use of an open-source software package, ProteoWizard [26], and then processing of mzXML files using XCMS online [27] resulted in time-aligned ion features, which are defined as a unique m/z at a unique retention time. Parameter settings for XCMS processing were as follows: centWave for feature detection ($m/z = 2.5$ ppm, minimum peak width = 5 s, and maximum peak width = 20 s); obiwrap settings for retention-time correction (profStep = 0.1); and parameters for chromatogram alignment, including mzwid = 0.015, minfrac = 0.25, and bw = 5. The relative quantification of ion features was based on EIC (extracted ion chromatogram) areas. The ion feature list was downloaded and the ion features at retention time 4.0–35.0 min of positive data were used for further statistical analysis in SIMCA-P (version 13.0 Umetrics, Sweden).

RESULTS AND DISCUSSION

PCA of *Actaea* samples using chromatographic fingerprints

Fig. S1 in the Electronic Supplementary Material (ESM) shows the eluting profiles of black cohosh over the entire UHPLC run time recorded by UV, ESI positive mode and ESI negative mode. PCA was performed on the ion features described in “Data processing” section. Fig. 1A shows a two-dimensional score plot for the first two principal components (PC1 and PC2). The score plot provides a conceptual overview of the samples by explaining 63.3% of the total variance. A clear four-group separation was observed. *A. racemosa* could be distinguished from two Asian species *A. foetida* and *A. dahurica*, and the other two American species *A. pachypoda* and *A. podocarpa*. The three American *Actaea* species are clearly separated from the two Asian ones. The corresponding loading plot (Fig. 1B) reflects how much each of the variables contributes to the classification. The variables V1–V9 are far away from the origin, indicating they contribute significantly to the separation, among which, V6–V9 with even-numbered m/z value are positioned to the right of the loading plot, and accordingly the two Asian *Actaea* species with relatively high level of these variables stay in the right part of the score plot.

The variables V1–V5 have been identified as cimicifugic acid F, cimicifugic acid B, fukinolic acid, cimicifugic acid A, cimicifugic acid E, respectively, in our previous study on flow injection mass spectrometric differentiation method for black cohosh (by ESI negative mode) [19]. The HPLC-UV fingerprint of them has been used to distinguish *A. racemosa* from major American and Asian *Actaea* species [11, 21]. The current study demonstrated that the EIC profile in MS positive mode of these five hydroxycinnamic acid esters could also distinguish *A. racemosa* from the other two American species and two Asian adulterants successfully, as shown in Fig. S2 (see ESM).

The variables V6–V9 are rarely mentioned in the literature on black cohosh authentication. Their even-numbered m/z value indicated that they probably are nitrogen containing compounds. The variable line plot of V6 and V7 revealed clearly their higher content levels in the two Asian species compared with the three American species (Fig. 2), which agreed with the EICs for V6 (V8, isomer of V6) and V7.

Putative Identification of Discriminative Compounds

The structural characterization of the discriminatory variables relies on HRAM values, retention times, MS²–MS⁵ mass spectra, ring-plus-double-bond (RDB) equivalent, literature reports and the use of standards when available.

The spectrometric and chromatographic evidence of the ions with even-numbered m/z value pointed to a class of compounds, the hydroxycinnamic acid amide (HCAA) glycosides. HCAAs are a diverse group of nitrogenous compounds found in higher plants [28]. Generally speaking, HCAAs are conjugates of hydroxycinnamic acid and amine via amide bonds. They occur as basic or neutral forms. In the basic forms, only one amine group of an aliphatic di- or poly-amine is linked with a hydroxycinnamic acid. In neutral forms, type 1, each terminal amine group of an aliphatic amine is neutralized by a hydroxycinnamic acid. In neutral forms, type 2, they are aromatic amines, instead of aliphatic amines, conjugated with hydroxycinnamic acid [29]. The *Actaea* HCAAs belong to the type 2 neutral form. In contrast to triterpenes and phenolic acids/esters, HCAAs from *Actaea* species have received far less attention. Only six HCAA glycosides including Cimicifugamide [30], isocimicifugamide [31], trans-feruloyl-tyramine 4-*O*- β -D-alloside [32], trans-feruloyl-(3-*O*-methyl) dopamine 4-*O*- β -D-alloside [32], trans-feruloyl tyramine 4-*O*- β -glucopyranoside [33], and cimicifugamide A [33] have been isolated from Asian *Actaea* species (Fig. 3).

Three authentic HCAAs, *trans*-*N*-caffeoyltyramine, *trans*-*N*-*p*-coumaroyltyramine, and *trans*-*N*-feruloyltyramine, were used as reference compounds to investigate the fragmentation behaviors in ESI positive mode on our platform. The spectra of *trans*-*N*-caffeoyltyramine and *trans*-*N*-feruloyltyramine (ESM Fig. S3) demonstrated consistent fragmentation patterns. Accurate mass measurement MS and MS² spectra showed that the cleavage of the amide bond to eliminate the amine moiety is preferred, and produces a predominant ion corresponding to the hydroxycinnamoyl group, which is in accordance with previous reports [34, 35]. The amine moiety could be determined by the exact neutral loss value. The carboxylic acid portion was able to be further confirmed by the characteristic ions in the subsequent multi-stage fragmentation. The caffeoyl moiety yielded ions of m/z 163, 145, 135, 117, and 89 (ESM Fig. S3A); whereas feruloyl/isoferuloyl group was characterized by product ions at m/z 177, 145, 117, and 89 (ESM Fig. S3B) [36].

In Fig. S4A (see ESM), compound V6 presents the protonated molecular ion at m/z 506.2023 (C₂₅H₃₂NO₁₀, 0.449ppm) and HCAA aglycone ion at m/z 344.1497 by eliminating a hexosyl moiety (162.0526 Da, very close to the theoretical value 162.0528 Da). The HCAA aglycone ion underwent further fragmentation similar to that observed in the reference compound *trans*-*N*-feruloyltyramine (Fig. S3B). The ion at m/z 177 originated from the loss of 167.0947 Da, corresponding to 3-methoxytyramine (Table 2). The fragment ions of m/z 177, 145, 117 indicated the feruloyl/isoferuloyl moiety. Nikolic et al.[36] distinguished between feruloyl and isoferuloyl based on the presence of a low abundance but diagnostic fragment ion at m/z 163, which was observed only during the fragmentation of protonated isoferulic acid, but not ferulic acid. By this method, the carboxylic acid portion was assigned as ferulic acid due to the absence of ion at m/z 163.

Considering the six HCAA glycosides isolated from *Actaea* plants are all glycosylated on the acid portion (4-*O*-glycosidation, Fig. 3), the position of glycosylation was assigned to the hydroxyl group on the feruloyl portion. The neutral loss of the amino moiety and the hexosylated feruloyl acylium ion at m/z 339 further supported the proposed assignment. Thus, V6 was partially characterized as feruloyl methyldopamine 4-*O*-hexoside. V8 was an isomer of V6 based on the almost identical spectra (data not shown) as V6. Three isomeric form of V6 have been isolated from genus *Actaea*, as shown in Fig. 3.

The full scan mass spectrum of V7 (ESM Fig. S4B) gave the $[M+H]^+$ ion at m/z 476.1917 ($C_{24}H_{30}NO_9$, 0.192ppm) and its dimer ion $[2M+H]^+$ at m/z 951.3757. In the MS^2 spectrum of the protonated molecular ion, the base peak at m/z 314 was produced by loss of 162 Da (the exact mass neutral loss was 162.0528 Da) corresponding to a hexosyl moiety. In *Actaea* species, the presence of glucose, galactose, and allose was reported to be attached to phenol group on the hydroxycinnamoyl portion (4-*O*-, Fig. 3) [30–33]. Further fragmentation of the $[M+H-hexosyl]^+$ ion at m/z 314 produced a predominant ion at m/z 177 in MS^3 spectrum, together with the ions at m/z 145 and 117 observed in the successive MS^4 and MS^5 spectra, thus a feruloyl group could be deduced. The exact neutral loss from m/z 314 to 177 was 137.0841 Da, which was ascribed to tyramine (Table 2) [34]. Therefore, compound V7 can be putatively assigned as feruloyl tyramine 4-*O*-hexoside. Two isomers have been isolated from *Actaea* plants (Fig. 3).

The EIC of ions at m/z 492 provided very interesting results, as seen in Fig. 4A. Three more major peaks were obtained other than compound V9. From their high resolution full scan spectra and the fragments of ions at m/z 492 (Fig. 5), compound V9, V9b, and V9c are isomers of feruloyl dopamine-*O*-hexosides, and V9a is feruloyl dopamine-*O*-di-hexoside. However, the determination of the glycosidation position is a very challenging task. Although the isolated hexosides of HCAA from *Actaea* plants were found to be 4-*O*-hexosides exclusively, both 4-*O* and 4'-*O*-hexosides have been described in other plants [37]. The hexosylated acylium ion of ferulic acid at m/z 339 and its water-loss ion at m/z 321 of V9b and V9c are evidence of 4-*O*-hexosides; but their lack of presence can not be the proof of 4'-*O*-hexosides in V9. Based on chemotaxonomic considerations, V9 had a great chance to be 4-*O* rather than 4'-*O*-hexosides. The presence of an ion at m/z 177 and absence of the diagnostic ion at m/z 163 strongly suggested that these compounds are amides of ferulic acid and not isoferulic acid, as mentioned above. Therefore, compound V9, V9b, and V9c can be partially characterized as feruloyl dopamine 4-*O*-hexoside, Cimicifugamide A and/or its isomers.

Compound V9a possessed the $[M+H]^+$ ion at m/z 654.2395 ($C_{30}H_{40}NO_{15}$, RDB 11.5, 0.389ppm) with an additional 162.0528 mass shift (compared to V9, V9b, and V9c) and $[M+Na]^+$ ion at m/z 676.2220 ($C_{30}H_{39}NO_{15}Na$, RDB 11.5, 1.197ppm). The product ion at m/z 492.1865 formed during in-source fragmentation and its further fragmented product ion at m/z 330, 177 supported the deduction that V9a was a dihexoside of ferulic acid amide. So V9a was assigned as feruloyl dopamine 4, 4'-*O*-dihexoside. To the best of our knowledge, this is the first description of a HCAA dihexoside in plant. This assignment was in accordance with the elution behavior that V9a eluted first due to its higher polarity caused by one more sugar moiety.

Marker Compounds Selection for Black Cohosh Authentication

As indicated in the PCA loading plot (Fig. 1A), V1–V9 contributed to the separation between *Actaea* clusters and could be marker candidates for the authentication of black cohosh. In this study, PLS-DA models were constructed using whole and four reduced datasets which comprised selected variables: 1) V1–V9, V9a, V9b, and V9c (dataset A); 2) V1–V5 (dataset B); 3) V6–V8 (dataset C); 4) V9, V9a, V9b, and V9c (dataset D). The plant samples were grouped into two classes: black cohosh (BC) and black cohosh adulterant/contaminant (BCA). A cross-validation method that leaves one sample out was used to evaluate the model and the prediction rate (the number of samples used to build the model that are correctly classified) was calculated based on the class of BC and BCA, respectively. Then the discrimination models were used to predict the class membership of new samples, the supplements samples, yielding prediction rate of test samples, i.e. the number of independent samples that are correctly classified.

The PLS-DA model built on whole dataset (Model W) and four reduced datasets (Model A–D) were evaluated by prediction rate of plant materials BC/BCA and black cohosh supplements, shown in Table 3. The Model A and D achieved equivalent result as Model W, producing 100% accuracy, which means the taxonomic origin of plant samples were correctly assigned to their own class (BC or BCA). The PCA score plot using dataset A (ESM Fig. S5A) are very similar with its whole dataset counterpart in Fig. 1A, demonstrating that those 12 variables could be representative of the whole dataset chosen in the study. Although only four variables were used, Model D can differentiate black cohosh from four other potential adulterants. The PCA score plot (ESM Fig. S5D) based on the four variables also demonstrates an isolated group of *A. racemosa*. The normalized intensity of V9 family members across *Actaea* species is shown in Fig. 4B. The distribution curves of two Asian species showed similar trends, so did the two American species, whereas the black cohosh demonstrated a completely different tendency. It is interesting to compare Model A with Model D, since both provide 100% accuracy to classify the training and test samples into BC or BCA group. Obviously, the method using V9 family is much more convenient with fewer variables and only one *m/z* value (492) involved. However, dataset A applies when the differentiation of each individual American species is also the interest of the study and not just authentication.

The prediction rate of Model B on BC and Model C on BCA (Table 3) were not satisfactory; indicating that authentication of black cohosh could not solely rely on the combination of V1–V5 or V6–V8, although some of those variables could be used as indicators of certain species. PCA score plots using dataset B and dataset C are shown in Figs. S5B and S5C (see ESM), some of *A. racemosa* samples were observed to overlap with *A. dahurica* and *A. podocarpa*, respectively.

The supplement samples were classified correctly as BC by all five models in the current collection of samples, pointing out the possibility of applying the method to supplements authentication. Further investigation will be carried out to verify or improve this approach with more supplements samples.

Conclusions

An HRAM LC-MS fingerprinting method combined with PCA was employed to search for new potential black cohosh authentication marker compounds. Five hydroxycinnamic acid esters (V1–V5, i.e. cimicifugic acid F, cimicifugic acid B, fukinolic acid, cimicifugic acid A, cimicifugic acid E) and seven HCAA glycosides (V6–V9 family) stood out from the loading plot. PLS-DA models were built on the whole dataset and four combinations of those marker candidates and the prediction rate were calculated. Models based on dataset A (V1–V9 family) and dataset D (V9 family only) achieved 100% prediction rate of BC and BCA in our current collection of both plant materials and dietary supplements. Model D, requiring only one *m/z* value at 492 (corresponding to four feruloyl dopamine-*O*-hexosides), is recommended as a faster and easier approach to authenticate black cohosh. HCAA glycosides were firstly proposed to be used as black cohosh authentication marker compounds and feruloyl dopamine 4, 4'-*O*-dihexoside (V9a) was described in plants for the first time. Using V9 family ions to differentiate *A. racemosa* from its common adulterant, the two Asian species, and its common contaminants, the two American black cohosh species, is quick, easy and robust since it is not economically to add or extract a member of V9 family for adulteration purposes. Thus, they can be used as effective marker compounds as orthogonal validation for authentication of Black Cohosh, or even used by themselves if necessary.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

(A) PCA-score plot based on HRAM ESI positive data from all *Actaea* samples listed in Table 1. Sample icons: ○ dahurica (da), △ foetida (fo), □ pachypoda (pa), ☆ podocarpa (po), and ☆ racemosa (ra, i.e. black cohosh). (B) PCA-loading plots for samples of the five *Actaea* species.

Fig. 2.

Line plot of variable V6 (blue square) and V7 (green circle) for all the *Actaea* samples in Table 1. Axis labels: da=dahurica; fo=foetida; pa=pachypoda; po=podocarpa; ra=racemosa. Extracted ion chromatograms for V6–V8 from five *Actaea* species were shown as references.

trans-Feruloyl-(3-O-methyl)dopamine 4-O- β -D-galactopyranoside
trans-Feruloyl-(3-methoxy)tyramine 4-O- β -D-galactopyranoside

trans-Feruloyl tyramine 4-O- β -D-allopyranoside

trans-Feruloyl tyramine 4-O- β -D-glucopyranoside

trans-Feruloyl-(3-O-methyl) dopamine 4-O- β -D-allopyranoside
trans-Feruloyl-(3-methoxy) tyramine 4-O- β -D-allopyranoside

trans-Feruloyl-(3-O-methyl) dopamine 4-O- β -D-allopyranoside
trans-Feruloyl-(3-methoxy) tyramine 4-O- β -D-allopyranoside

trans-Feruloyl dopamine 4-O- β -D-galactopyranoside

Fig. 3.

Structures of hydroxycinnamic acid amide (HCAA) glycosides isolated from genus *Actaea*.

Fig. 4.

A) Extracted ion chromatograms (EICs) for ion at m/z 492.18–492.19 of five *Actaea* species. B) Comparison of normalized intensity of V9a, V9, V9b, and V9c across species. Error bars represent standard deviations. Legends: da=dahurica; fo=foetida; pa=pachypoda; po=podocarpa; ra=racemosa.

Fig. 5. Full scan (HRAM) and MS² mass spectra of ions at *m/z* 492 from compound V9a, V9, V9b and V9c. Abbreviations: hex=hexosyl; dop=dopamine.

Table 1*Actaea* samples used in this study

ID	Species	Source
BCR01	racemosa	AHP
BCR02	pachypoda	AHP
BCR03	foetida	AHP
BCR04	racemosa	AHP
BCR05	pachypoda	AHP
BCR06	racemosa	AHP
BCR07	racemosa	AHP
BCR08	racemosa	AHP
BCR09	racemosa	AHP
BCR10	podocarpa	AHP
BCR11	podocarpa	AHP
BCR12	podocarpa	AHP
BCR13	foetida	AHP
BCR16	racemosa	AHP
BCR17	racemosa	AHP
BCR18	foetida	AHP
BCR19	foetida	AHP
BCR20	foetida	AHP
BCR21	foetida	AHP
BCR22	foetida	AHP
BCR23	foetida	AHP
BCR24	foetida	AHP
SRM3295	racemosa	NIST
BCA06	dahurica	Hebei, China
BCA07	dahurica	Sichuan, China
BCA08	dahurica	North Korea
BCS01	supplement	Manufacture A
BCS02	supplement	Manufacture B
BCS03	supplement	Manufacture C
BCS04	supplement	Manufacture D
BCS05	supplement	Manufacture E

Table 2

Typical neutral loss of amine moiety and cation of hydroxycinnamyl moieties in HCAAs

	Name & Formula	Structure	Accurate mass value
Amine moiety (neutral loss)	Tyramine $C_{18}H_{11}NO$		137.0841
	Dopamine $C_{18}H_{11}NO_2$		153.0790
	3-Methoxytyramine/3- <i>O</i> -methyldopamine		167.0946
Hydroxycinnamoyl group (cation)	Caffeoyl $C_9H_7O_3^+$		163.0390
	Feruloyl $C_{10}H_9O_3^+$		177.0546
	Isoferuloyl $C_{10}H_9O_3^+$		177.0546

Table 3

PLS-DA models using whole and four reduced datasets

PLS-DA Model	Variables Used	Prediction Rate ^a		
		BC ^b	BCA ^c	Supplement ^d
Model W	Whole dataset	100.0%	100.0%	100.0%
Model A	V1–V9, V9a, V9b, V9c	100.0%	100.0%	100.0%
Model B	V1–V5	65.0%	100.0%	100.0%
Model C	V6–V8	100.0%	81.3%	100.0%
Model D	V9, V9a, V9b, V9c	100.0%	100.0%	100.0%

^a the number of samples that are correctly classified.

^b *Actaea racemosa* plant samples, used as training samples.

^c other *Actaea* plant samples, used as training samples.

^d used as test samples.