IS THIS REALLY BLACK COHOSH? AUTHENTICATION OF BLACK COHOSH PRODUCTS USING UPLC-QTOF-MS CHEMICAL PROFILING

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HIGHLIGHTS

- **Authentication and chemical identification of different *Actaea* species (black cohosh) using novel analytical and informatics tools.**
- **Demonstrated the comprehensive workflow of Progenesis QI for chemical profiling of large LC/MS datasets**
- **The application of UPLC-QTOF-MS for multivariate analysis of black cohosh is a powerful approach in the authentication of black cohosh species and commercial products**

INTRODUCTION

Black cohosh (*Actaea racemosa* L.) is a native North American species, where it is widely used as an herbal supplement for the relief of menopausal symptoms. It is available in Europe as a phytomedicine since the 1950s[1]. Black cohosh commercial products were ranked in the top ten of supplement sales in the USA. In addition to *A. racemosa,* there are other North American and Asian *Actaea* species. Due to the continual increase in sales and collection of *A. racemosa*, mix up and adulteration with related and non-related species could be seen in the market. This is a major concern as it poses health issues to the unknowing consumers.

Morphological examination to genetic testing have been commonly used to authenticate different *Actaea* species. Studies using instruments such as HPTLC, HPLC-DAD, HPLC-ELSD, GC-MS, NMR, and LC-MS have also been used to profile these species. Many of the studies have focused on identifying key chemical components[2]. A newer approach has been the development of untargeted methods to compare complete chemical profile using authenticated references.

In the present study, a multivariate statistical method using UPLC-QTof -MS was used for the classification of different *Actaea* species and commercial black cohosh products.

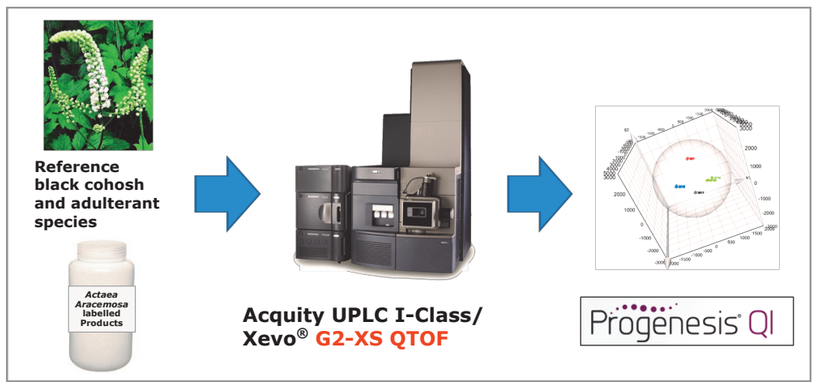


Figure 1. *The metabolomics workflow for the black cohosh study. The reference black cohosh samples and commercial products were extracted and analyzed using an Acquity UPLC-I Class with Xevo G2-XS QTOF for LC/MS data acquisition. The data was then imported into Progenesis QI (Nonlinear Dynamics, Newcastle, UK) for multivariate analysis and identification of key markers.*

METHODS

SAMPLE INFORMATION AND PREPARATION

- 38 samples [22 reference samples (roots and rhizomes)] and 16 commercial black cohosh products (roots and rhizomes).
- Weigh about 2.5 grams of the ground sample (roots and rhizomes) and extracted with 80% methanol.
- Vortex at 3000 rpm (30 mins) and centrifuge at 4000 rpm (10 min). Repeat extraction 2X
- Pool the supernatant and evaporate under N_2 at 25-30°C
- 10mg of extract was dissolved in 80% methanol (1ml) and 25x dilution was done.
- Create QC sample (0.1ml of each sample).

INSTRUMENTAL CONDITIONS

LC CONDITIONS:

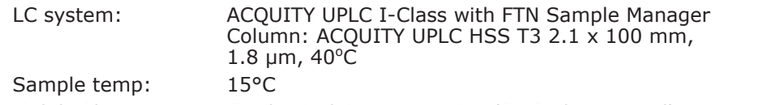
LC system: ACQUITY UPLC I-Class with FTN Sample Manager
Column: ACQUITY UPLC HSS T3 2.1 x 100 mm, 1.8 µm, 40°C
Sample temp: 15°C
Mobile Phase: Gradient elution, A: Water (0.1% formic acid) & B: acetonitrile

Time	Flow	%A	%B	Curve
0	0.600	99	1	Initial
0.5	0.600	99	1	6
16	0.600	65	35	6
18	0.600	0	100	1
20	0.600	99	1	1

MS CONDITIONS:

MS system: Xevo G2-XS QTof MS
Acquisition range: 50-1500 Da (0.1s scan rate)
Acquisition mode: MS^E, ESI^- and ESI^+ in resolution mode
Capillary voltage: 3.0 kV (ESI^+)/2.5 kV (ESI^-)
Cone voltage: 30 V
Collision energy (eV): low CE: 6/High CE: 15-45eV
Source temp: 120°C, Desolvation temp: 550°C
QC was checked to ensure system suitability throughout the data acquisition.

DATA ANALYSIS:



The black cohosh data was analyzed using a novel data processing and statistical tool called Progenesis QI v2.2 (Nonlinear Dynamics, Newcastle, UK). With Progenesis QI, users can quickly perform differential analysis of results across different sample groups thereby facilitating identification and quantitation of potential biomarkers. The Progenesis QI software adopts an intuitive step-by-step workflow to perform comparative high resolution UPLC-MS metabolomics. For the multivariate analysis, Ezinfo V3.03 (Umetrics) is used for statistical modelling and is integrated with Progenesis.

RESULTS AND DISCUSSION

UPLC-QTOF-MS Ion Intensity Map

Figure 2A) shows the UPLC-QTOF-MS chromatogram on an *Actaea racemosa* sample. All samples were spectral aligned to ensure correct comparison between samples. All samples were then converted into an ion intensity map (Figure 2B) where the peaks were picked and normalized to all compounds using a reference sample. This 3D maps were then used for multivariate modeling. All processing steps are part of the Progenesis QI workflow.

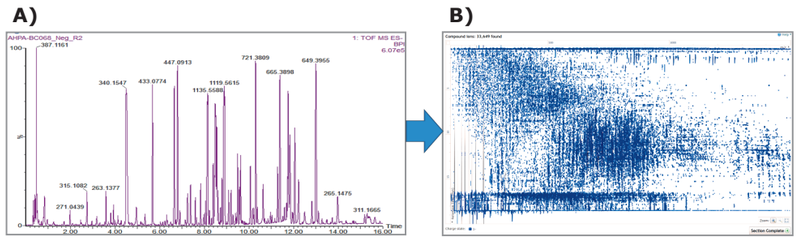


Figure 2. A) *An LC/MS chromatogram of Actaea racemosa* ***B)*** *An LC/MS ion intensity map of Actaea racemosa with 33,649 peaks selected. This chemical fingerprint is created for all the samples and used for multivariate analysis in Progenesis QI.*

Principal Component Analysis (PCA)

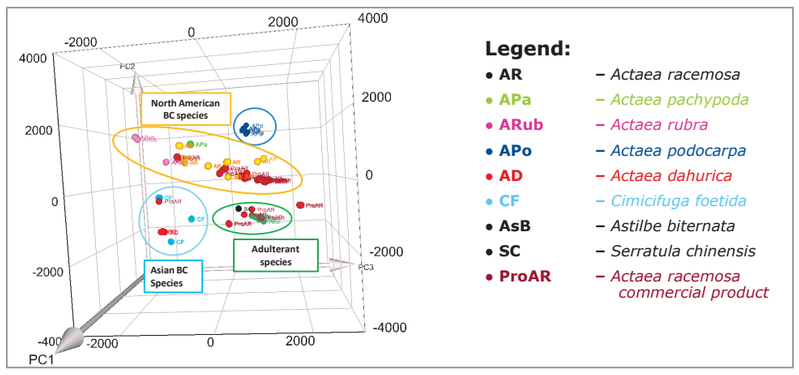


Figure 3. *3D PCA scores of all reference black cohosh samples with commercial black cohosh products.*

Figure 3 shows a 3D PCA scores plot of all the black cohosh reference species with the commercial black cohosh products. Three North American species (*A. racemosa*, *A. pachypoda* and *A. rubra*) cluster close together as they commonly grow in similar geographies and can have similar chemical profiles. The other North American species, *A. podocarpa* clustered further away from the three North American species and is known to grown in isolated areas. The Asian black cohosh species (*A. dahurica and C.foetida*) are two commonly substituted species for *A. racemosa* and from their LC/MS profiles are significantly separated from the North American species. The last two species, *A. biternata* and *S. chinensis* are two common economic adulterants for black cohosh and show unique clusters in the PCA scores. Overall, the PCA scores of the LC/MS chemical profiles show how distinct the black cohosh species and its adulterants are from each other. For the 16 black cohosh labeled commercial products (ProAR), 11 of the 16 were clustered with the authentic *A.racemosa* species. All other samples were seen to group with other species of black cohosh (*C. foetida*) or common adulterants such as *S.chinensis* and *A.biternata*. This results shows how common adulteration of black cohosh are detected in the market and the sensitivity of LC/MS to quickly differentiate which correct black cohosh species is in the commercial products.

Class Discrimination of Black Cohosh Species using Partial Least Squares Analysis–Discriminant Analysis (PLS-DA)

To evaluate the prediction capability of authenticating the black cohosh commercial products, a PLS-DA model (Figure 4) was generated using the LC/MS chemical profiles of the reference black cohosh species and adulterants. The model generated 12 components with variance explained, R^2=98% and variance predicted, Q^2=86%. Each reference black cohosh sample was then tested by classifying it independently in the PLS-DA model. From the classification results (not shown), all the reference black cohosh samples and adulterants were successfully classified into their respective groups with no misclassifications observed. This result explain how distinct the LC/MS black cohosh chemical profiles were from each other and the potential to be used for species identification.

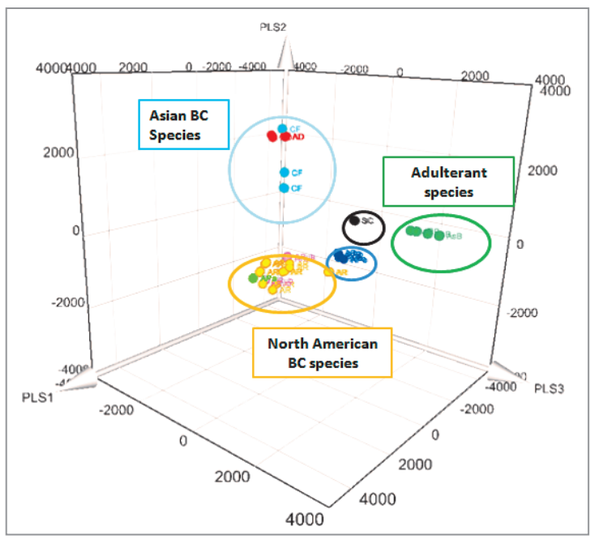


Figure 4. ***A)*** *PLS-DA scores plot of all the reference black cohosh and adulterant species. Total variance explained, 49.3%.*

Classification of *Actaea racemosa* commercial products

With the PLS-DA model validated, all the *A. racemosa* commercial samples were predicted using the PLS-DA model of the authentic references. From the table of results (bottom), There were 11 of the 16 products that were identified to contain *A. racemosa* while 3 contained the Asian black cohosh species (*C.fotieda* and *A.dahurica*), 1 was identified as the adulterant species, *S.chinensis* and 1 was identified as *A.biternata* or *A.dahurica.* Three products (BC 6,10 and 11) were considered weakly classified and should be further investigated.

Commercial Sample	Condition	Classification
AHPA-BC001_Neg_R1	ProAR	CF
AHPA-BC002_Neg_R1	ProAR	AR
AHPA-BC003_Neg_R1	ProAR	AR
AHPA-BC004_Neg_R1	ProAR	AR
AHPA-BC005_Neg_R1	ProAR	AR
AHPA-BC006_Neg_R1	ProAR	AsB/AD?
AHPA-BC007_Neg_R1	ProAR	AR
AHPA-BC008_Neg_R1	ProAR	AR
AHPA-BC009_Neg_R1	ProAR	AR
AHPA-BC010_Neg_R1	ProAR	SC?
AHPA-BC011_Neg_R1	ProAR	AR?
AHPA-BC012_Neg_R1	ProAR	CF
AHPA-BC013_Neg_R1	ProAR	AR
AHPA-BC014_Neg_R1	ProAR	AR
AHPA-BC015_Neg_R1	ProAR	AR
AHPA-BC016_Neg_R1	ProAR	AD

Identification of key markers from PLS-DA

To identify the key markers for each species, the PLS-DA loadings were obtained by using the 3D scores plot of the reference and extracting the key m/z values for further investigation. By using two different dimensions (Figure 5), unique markers can be obtained for each of the different species.

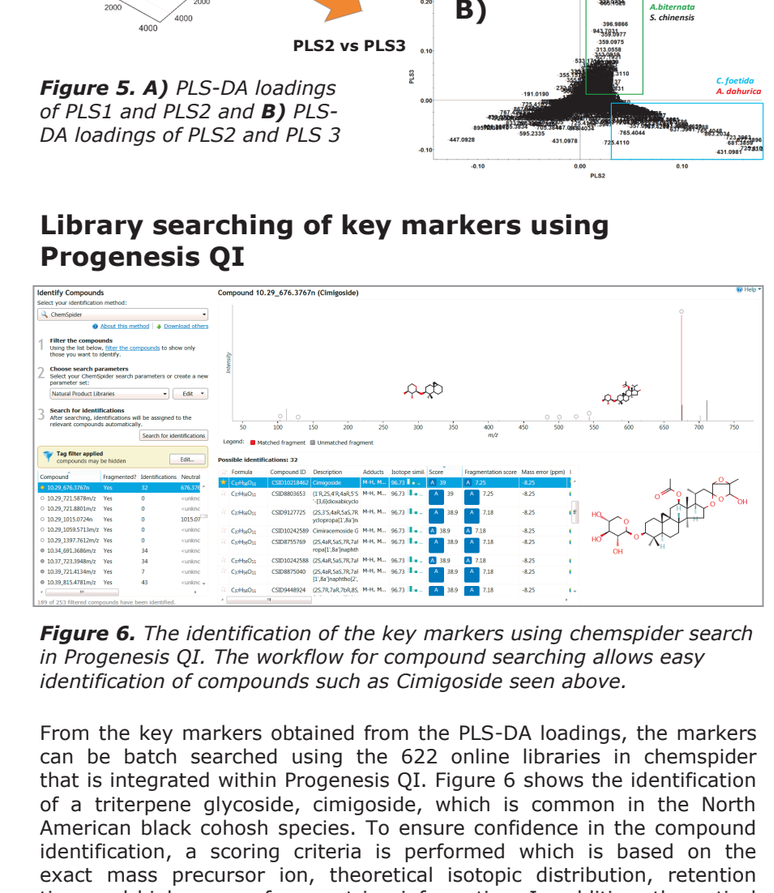


Figure 5. A) *PLS-DA loadings of PLS1 and PLS2 and* ***B)*** *PLS-DA loadings of PLS2 and PLS 3*

Library searching of key markers using Progenesis QI

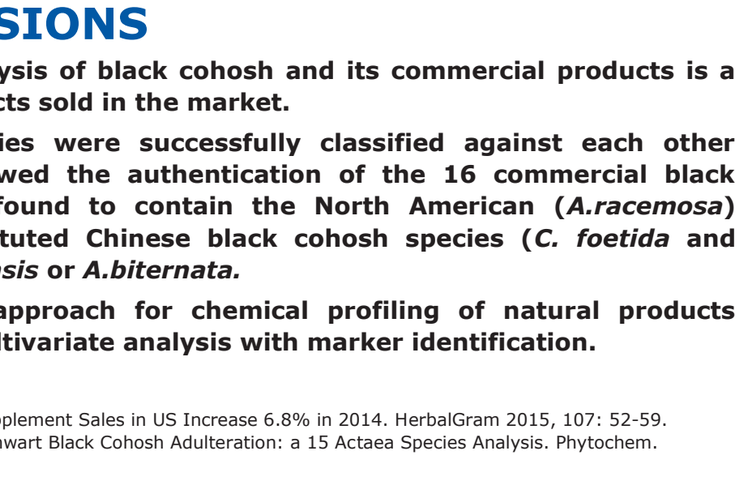


Figure 6. *The identification of the key markers using chemspider search in Progenesis QI. The workflow for compound searching allows easy identification of compounds such as Cimigoside seen above.*

From the key markers obtained from the PLS-DA loadings, the markers can be batch searched using the 622 online libraries in chemspider that is integrated within Progenesis QI. Figure 6 shows the identification of a triterpene glycoside, cimigoside, which is common in the North American black cohosh species. To ensure confidence in the compound identification, a scoring criteria is performed which is based on the exact mass precursor ion, theoretical isotopic distribution, retention time and high energy fragment ion information. In addition, theoretical fragmentation of a candidate compounds was performed and then matched to the resulting '*in silico*' fragmentation against the measured fragments for a compound. Further research is currently being done to identify all the key markers for each of the black cohosh and adulterant species.

CONCLUSIONS

- **The application of UPLC-QTOF-MS for multivariate analysis of black cohosh and its commercial products is a powerful approach for authenticating commercial products sold in the market.**
- **All the reference black cohosh and adulterant species were successfully classified against each other using their LC/MS chemical profiles. This result allowed the authentication of the 16 commercial black cohosh species. Only 11 of the 16 products were found to contain the North American (*A.racemosa*) specie while the other 5 contained either the substituted Chinese black cohosh species (*C. foetida* and *A. dahurica*) or the common adulterant species, *S.chinensis* or *A.biternata.***
- **Progenesis QI provides a comprehensive workflow approach for chemical profiling of natural products from processing large LC/MS datasets to conducting multivariate analysis with marker identification.**

REFERENCES

1. Smitha T, Lynch ME, Johnson J, Kawa K, Bauman H, Blumenthal M. Herbal Dietary Supplement Sales in US Increase 6.8% in 2014. HerbalGram 2015, 107: 52-59.
2. Jiang B, Ma C, Motley T, Kronenberg F, Kennelly EJ. Phytochemical Fingerprinting to Thwart Black Cohosh Adulteration: a 15 Actaea Species Analysis. Phytochem. Anal. 2011, 22: 339–351.

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