# Characterization of Steroidal Saponins From Two *Dioscorea* Species Using Liquid Chromatography and the Agilent 6500 Series Accurate-Mass Q-TOF LC/MS

Application Note

Food Testing & Agriculture


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## Abstract

A fast and effective UHPLC/QTOF-MS method using a simple methanol extraction has been developed to determine the structural characteristics of steroidal saponins from dried rhizomes of two *Dioscorea* species (*D. villosa* L. and *D. cayenensis* Lam.) and dietary supplements. The saponins were identified or tentatively characterized from their retention times and mass spectra and the fragmentation patterns of reference standards. Statistical analysis demonstrates that saponin content can be used to distinguish the two species and suggests the identification of species used in dietary supplements.


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## Introduction

Several species of the genus *Dioscorea*, commonly known as yam, provide an important foodstuff in Africa, Asia, and tropical America. The genus consists of approximately 630 species distributed throughout the world. Most of these species contain steroidal saponins that have a wide range of pharmacological activities [1]. Diosgenin, which is obtained by hydrolysis of yam saponins, is used as starting material for the synthesis of steroidal drugs, including progesterone and testosterone. More than 50 steroid saponins have been derived and characterized from *Dioscorea* species. They are the primary physiologically active compounds found in yam [2,3].

Detailed characterization of the steroidal saponins from several of these species could facilitate full usage these plants as sources for pharmacologically important compounds. LC/MS has proven to be a very convenient and efficient technique for the identification of steroidal saponins from plant extracts [4-8]. However, the combination of ultra-high performance liquid chromatography (UHPLC) with quadrupole time-of-flight mass spectrometry (Q-TOF MS) offers significant advantages for rapid screening of target compounds in complex matrixes such as these plant species. This approach negates the need for laborious and tedious purification of compounds from crude extracts.

This application note describes a recently published study with the goal of characterizing saponins from *D. villosa* and *D. cayenensis* using UHPLC/Q-TOF MS [1]. Fragmentation patterns were carefully detailed, and enabled the detection of 21 steroidal saponins with six aglycone skeletons from crude extracts. Seventeen were identified by comparison of retention times, single MS, and MS/MS with reference standards. This method also enabled the rapid differentiation of the two species with high reproducibility and sensitivity.

## Experimental

### Samples

Rhizomes and roots of *Dioscorea villosa* L. (NCNPR accession # 9800) and *D. cayenensis* Lam. (NCNPR accession # 9166, 9462) were obtained from the living collection of the NCNPR Maynard W. Quimby Medicinal Plant Garden, The University of Mississippi, as described, as were commercially available root powders and dietary supplements claiming to contain *D. villosa* [1].

### Reagents and standards

Chemicals and steroidal saponin standards were obtained and used as described [1].

### Instruments

This study was performed on an Agilent 1290 Infinity LC System equipped with a G4220A autosampler, a G4220A binary pump, and a G1316C Thermal Column Compartment coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS equipped with an ESI source with Agilent Jet Stream technology. Reference solution was introduced into the ESI source through a T-junction using an Agilent 1200 Series G1310A ISO Isocratic Pump for the purposes of reference ion mass correction. The instrument conditions are listed in Table 1.

*Table 1. UHPLC and Q-TOF MS Run Conditions*

| **LC conditions** | | | |
|---|---|---|---|
| Column | Agilent Poroshell 120 EC-8, 2.1 × 100 mm, 2.7 µm (p/n 695775-906) | | |
| Column temperature | 35 °C | | |
| Injection volume | 2 µL | | |
| Mobile phase | A) 0.1% formic acid in water<br>B) 0.1% formic acid in acetonitrile | | |
| Flow rate | 0.25 mL/min | | |
| Gradient | Time (min) | % A | % B |
| | 0.00 | 75 | 25 |
| | 5.00 | 70 | 30 |
| | 7.00 | 55 | 45 |
| | 10.00 | 40 | 60 |
| | 15.00 | 0 | 100 |
| | 20.00 | 0 | 100 |
| Wash | 5 minutes with 100% B | | |
| Equilibration | 75% A/25% B, for 3 minutes | | |
| Total run time | 15 minutes | | |
| **MS conditions** | | | |
| Ionization mode | ESI in positive and negative ion | | |
| $N_2$ drying gas temperature | 250 °C | | |
| $N^2$ drying gas flow | 11 L/min | | |
| Nebulizer pressure | 35 psig | | |
| Sheath gas temperature | 325 °C | | |
| Sheath gas flow | 10 L/min | | |
| Capillary voltage | 3,500 V | | |
| Nozzle voltage | 0 V | | |
| Skimmer | 65 V | | |
| Oct RF voltage | 750 V | | |
| Fragmentor voltage | 100 V | | |
| Collision energy (for MS/MS experiments) | 30 eV | | |
| Mass range | 100–1,700 *m/z* | | |

### Standards and plant sample preparation

All saponin steroid standards were prepared at a concentration ranging from 0.5 to 10 µg/mL, and dry plant samples or dietary supplements were subjected to an exhaustive extraction with methanol as described [1].

## Results and Discussion

### UHPLC/Q-TOF analysis of *Dioscorea* saponins

Accurate mass data obtained from the Q-TOF enabled chemical formula identification of several steroidal saponins in single MS mode, and structural information when forming product ions in MS/MS. The Agilent Molecular Feature Extractor (MFE) was used to aid in the identification of the chemical formulae by resolving coeluting interferences, and grouping the isotopic clusters, all adducts, dimers, and trimers if present.

The majority of compounds were detectable in both negative and positive ion modes. Using MS in the negative mode gave clear $[M\text{-}H]^-$ or $[M+HCOO]^-$ ions, and MS and MS/MS in the positive mode gave sufficient information for structural elucidation. The key advantage offered by a Q-TOF instrument is its accurate mass measurement, which provides the elemental composition of parent and fragment ions. A threshold of 5 ppm accuracy, which is easily attainable using the Agilent 6500 series LC/MS Q-TOF, is widely accepted for determination of elemental compositions when combined with both isotope spacing and ratios. Accurate mass MS/MS, when combined with the characteristic retention time (RT) as compared to reference standards, affords highly reliable compound confirmation. The characteristic fragment ions provide crucial information for identification when reference standards are not available.

The optimized method was used to analyze 17 reference standards and identify four additional saponins, and an MS/MS spectral library was created using the data. Both positive and negative electrospray ionization modes were used to obtain the data at collision energies of 0, 10, 20, 30, and 40 eV. The fragmentation behavior was different for all 21 compounds, thus necessitating multiple collision energies. Only singly charged positive ($[M+H]^+$ or $[M+H–H_2O]^+$) and negative ions ($[M\text{-}H]^-$ or $[M+HCOO]^-$) were used to produce targeted MS/MS spectra.

Spectra generated from the standards were imported into the library building tool (Agilent MassHunter PCDL Manager, Version B.04.00) to generate the *Dioscorea* saponins library, and the utility of the MS/MS library was tested on all samples of *Dioscorea* species and five commercial products.

## Identification of steroidal saponins

Nine samples of *D. villos*a and three samples of *D. cayenensis* standardized plant material were used to develop a saponin fingerprint. Other samples of *Dioscorea* were then tested similarly to confirm the usefulness of the MS/MS library. All samples of *Dioscorea* used were listed in the previous publication [1]. The method was validated by testing a number of other samples within a single species of *Dioscorea*.

The base peak chromatograms (BPCs) of saponins from the *Dioscorea* extracts was generated using the UHPLC/QTOF-MS method in positive mode, and it revealed a total of 21 steroidal saponin compound peaks isolated from the two *Dioscorea* species (Figure 1, Table 2). Compounds 1–17 were identified by comparison of their retention times and MS spectra with those of the reference standards in the MS/MS library without ambiguity. Based on previous publications and our deduction, the other four saponins were identified or tentatively characterized in the crude extracts from *Dioscorea* species.

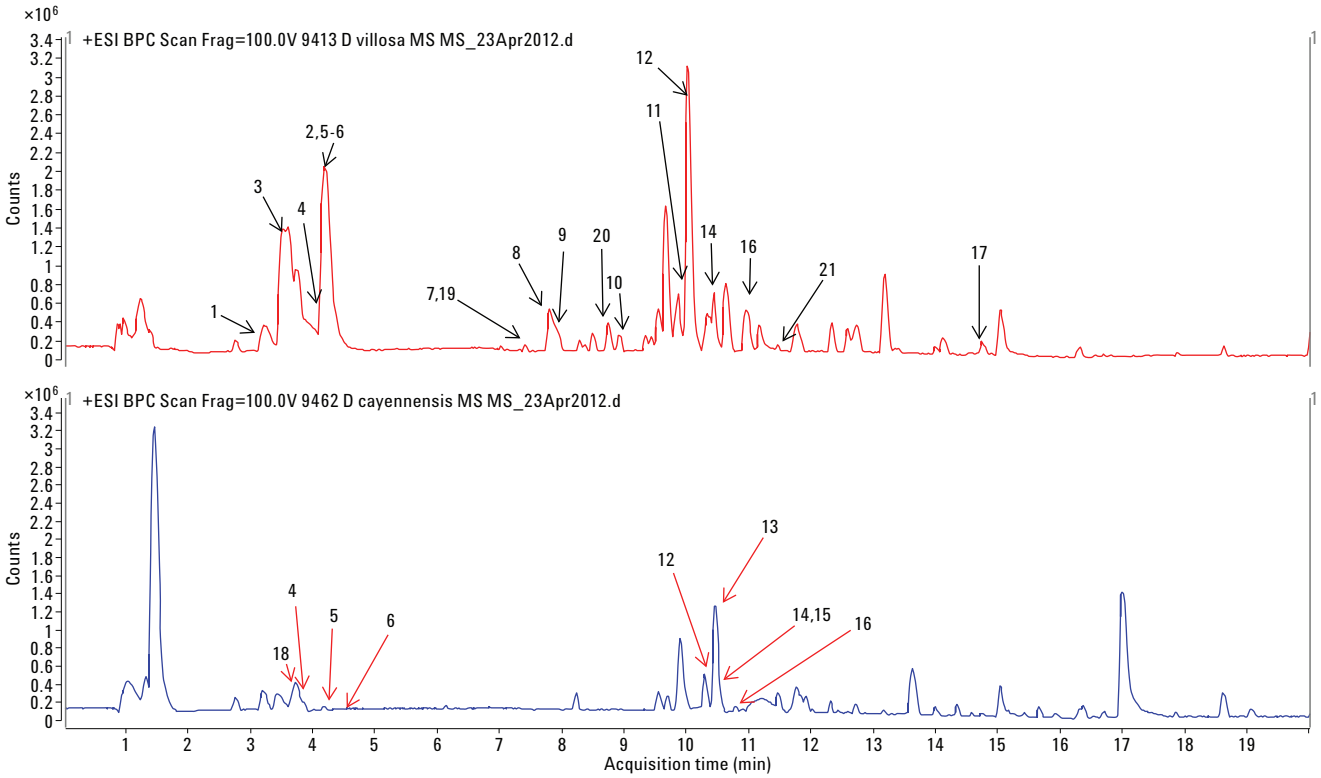


*Figure1. Base peak chromatograms (BPCs) of the D. villosa and D. cayenensis extracts analyzed by UHPLC/QTOF-MS in positive ESI mode.*

*Table 2. Accurate Mass Data for Steroidal Saponins Determined Using UHPLC/QTOF-MS*

| Peak | RT | Formula | Selected ions | *m/z* calculated | *m/z* experimental | Error (mDa) | Identities | Species |
|---|---|---|---|---|---|---|---|---|
| **Cholestane-type steroids** | | | | | | | | |
| 1 | 3.01 | $C_{39}H_{66}O_{14}$ | $[M+Na]^+$<br>$[M+HCOO]^-$ | 781.4350<br>803.4429 | 781.4348<br>803.4431 | 0.2<br>–0.2 | Dioscoreavilloside A | *D. villosa* |
| 2 | 4.3 | $C_{39}H_{64}O_{14}$ | [M+Na]+<br>$[M-H_2O+H]^+$<br>$[M+HCOO]^-$ | 779.4194<br>739.4269<br>801.4273 | 779.4194<br>739.4268<br>801.4273 | 0.0<br>0.1<br>0.0 | Dioscoreavilloside B | *D. villosa* |
| **Furostane-type steroids** | | | | | | | | |
| 3 | 3.61 | $C_{57}H_{94}O_{28}$ | $[M-H_2O+H]^+$<br>$[M-H]^-$ | 1209.5904<br>1225.5853 | 1209.5914<br>1225.5852 | –1.0<br>0.1 | Parvifloside | *D. villosa* |
| 4 | 3.96 | $C_{51}H_{84}O_{23}$ | $[M-H_2O+H]^+$<br>$[M-H]^-$ | 1047.5376<br>1063.5325 | 1047.5376<br>1063.5329 | 0.0<br>–0.4 | Protodeltonin | *D. villosa*<br>*D. cayenensis* |
| 5 | 4.15 | $C_{51}H_{84}O_{22}$ | $[M-H_2O+H]^+$<br>$[M-H]^-$ | 1031.5427<br>1047.5376 | 1031.5427<br>1047.5371 | 0.0<br>0.5 | Protodioscin | *D. villosa*<br>*D. cayenensis* |
| 6 | 4.66 | $C_{45}H_{74}O_{18}$ | $[M-H_2O+H]^+$<br>$[M-H]^-$ | 885.4848<br>901.4797 | 885.4850<br>901.4799 | –0.2<br>–0.2 | Protobioside | *D. villosa*<br>*D. cayenensis* |
| 7 | 7.56 | $C_{51}H_{82}O_{22}$ | $[M+H]^+$<br>$[M-H]^-$ | 1047.5376<br>1045.5219 | 1047.5376<br>1045.5220 | 0.0<br>–0.1 | Huangjiangsu A | *D. villosa* |
| 8 | 7.65 | $C_{51}H_{82}O_{21}$ | $[M+H]^+$<br>$[M-H]^-$ | 1031.5427<br>1029.5270 | 1031.5428<br>1029.5272 | –0.1<br>–0.2 | Pseudoprotodioscin | *D. villosa* |
| 9 | 7.83 | $C_{45}H_{72}O_{17}$ | $[M+H]^+$<br>$[M-H]^-$ | 885.4848<br>883.4691 | 885.4846<br>883.4694 | 0.2<br>–0.3 | 26-O-$\beta$-D-glucopyranosyl-3$\beta$, 26-diol-25(R)-furost-5,20(22)-dien-3-O-$\alpha$-L-rhamnopyranosyl(1→2)-O-$\beta$-D-glucopyranoside | *D. villosa* |
| **Spirostane-type steroids** | | | | | | | | |
| 10 | 9.21 | $C_{45}H_{72}O_{17}$ | $[M+H]^+$<br>$[M-H]^-$<br>$[M+HCOO]^-$ | 885.4848<br>883.4691<br>929.4746 | 885.4841<br>883.4672<br>929.4749 | 0.7<br>1.9<br>–0.3 | 25R-dracaenoside G | *D. villosa* |
| 11 | 10.05 | $C_{51}H_{82}O_{22}$ | $[M+Na]^+$<br>$[M-H]^+$ | 1069.5195<br>1045.5220 | 1069.5194<br>1045.5207 | 0.1<br>1.3 | Zingiberensis Saponin I | *D. villosa* |
| 12 | 10.39 | $C_{45}H_{72}O_{17}$ | $[M+H]^+$<br>$[M+Na]^+$<br>$[M-H]^-$ | 885.4848<br>907.4667<br>883.4691 | 885.4850<br>907.4664<br>883.4691 | –0.2<br>0.3<br>0.0 | Deltonin | *D. villosa*<br>*D. cayenensis* |
| 13 | 10.42 | $C_{51}H_{82}O_{20}$ | $[M+Na]^+$<br>$[M-H]^-$ | 1037.5297<br>1013.5321 | 1037.5295<br>1013.5323 | 0.2<br>–0.2 | Parrisaponin or Asperin | *D. cayenensis* |
| 14 | 10.59 | $C_{45}H_{72}O_{16}$ | $[M+H]^+$<br>$[M+HCOO]^-$ | 869.4899<br>913.4797 | 869.4895<br>913.4794 | 0.4<br>0.3 | Dioscin | *D. villosa*<br>*D. cayenensis* |
| 15 | 10.67 | $C_{45}H_{72}O_{17}$ | $[M+H]^+$<br>$[M+Na]^+$<br>$[M+HCOO]^-$ | 885.4848<br>907.4667<br>929.4746 | 885.4847<br>907.4669<br>929.4746 | 0.1<br>–0.2<br>0.0 | Gracillin | *D. cayenensis* |
| 16 | 10.97 | $C_{39}H_{62}O_{12}$ | $[M+H]^+$<br>$[M-H]^-$<br>$[M+HCOO]^-$ | 723.4320<br>721.4163<br>767.4218 | 723.4319<br>721.4146<br>767.4217 | 0.1<br>1.9<br>0.1 | Progenin III | *D. villosa*<br>*D. cayenensis* |
| 17 | 14.83 | $C_{27}H_{42}O_3$ | $[M+H]^+$ | 415.3212 | 415.3214 | –0.2 | Diosgenin | *D. villosa* |
| **Other compounds** | | | | | | | | |
| 18 | 3.83 | $C_{57}H_{94}O_{26}$ | $[M-H]^-$ | 1193.5961 | 1193.5970 | 0.7 | Asperoside | *D. cayenensis* |
| 19 | 7.22 | $C_{57}H_{92}O_{27}$ | $[M-H]^-$ | 1207.5753 | 1207.5741 | 1.0 | Zingiberenin G | *D. villosa* |
| 20 | 8.91 | $C_{39}H_{62}O_{13}$ | $[M+H]^+$ | 739.4263 | 739.4274 | –1.5 | Diosgenin diglucoside | *D. villosa* |
| 21 | 11.5 | $C_{39}H_{62}O_{12}$ | $[M+H]^+$ | 723.4314 | 723.4321 | 0.96 | Progenin II | *D. villosa* |

All peaks displayed the characteristic fragmentation patterns of steroidal saponins. Data obtained from all analyzed samples suggested that 18 compounds were present in *D. villosa* and nine compounds in *D. cayenensis*. Three compounds (13, 15, and 18) were found only in *D. cayenensis*. Eleven compounds (1–3, 7–11, 17, 19–21) were found in the majority of *D. villosa* samples, and six compounds (4–6, 12, 14, 16) were common to both *Dioscorea* species (Figure 1). While the saponin content of the *D. villosa* samples varied, with one containing only one compound (diosgenin, 17), all three samples of *D. cayenensis* contained the same nine saponins (4–6, 12–16, 18).

## Species differentiation

Principal component analysis (PCA) was performed in Agilent Mass Profiler Professional (MPP) software to compare and distinguish nine samples of *D. villosa*, three samples of *D. cayenensis*, and five dietary products claiming to contain *D. villosa*. Because Compounds 3, 7–11, 13, and 15 (Table 2) accounted for 91% of the variation in the data, they were used in the PCA analysis. The principal components (PCs) are displayed graphically as a scores plot (Figure 3), which is useful for detecting any groupings in the data set. The numerical value of a loading of a given variable of a PC represents the degree of commonality of that variable with that PC. In this case, separation of the variables was observed primarily in the first two principal components (PC-1 and PC-2). The scores and loading plots for PC-1 versus PC-2 showed clear separation of Compounds 3 and 7–11 from Compounds 13 and 15 (data not shown).

The majority of separation between *Dioscorea* species in Figure 2A was due to PC-1 (x-axis). Figure 2B illustrates how much variability between the two species was due to each principal component (PC). Four of the nine samples of *D. villosa* were distinguished in Figure 2A from the three *D. cayenensis* samples. *D. villosa* samples were closely clustered, except for one which was positioned apart due to specific patterns in the relative abundances of Compounds 8 and 9. The *D. cayenensis* samples were also closely clustered, but widely separated from the *D. villosa* samples. The five products tested clustered with the *D. villosa* samples, suggesting that they were derived from *D. villosa*. This PCA analysis using Compounds 3, 7–11, 13, and 15 illustrates that distinctions can be made between the two species. The sample size is too small to create a robust model that will confidently predict the species of unknowns but this does show its feasibility.

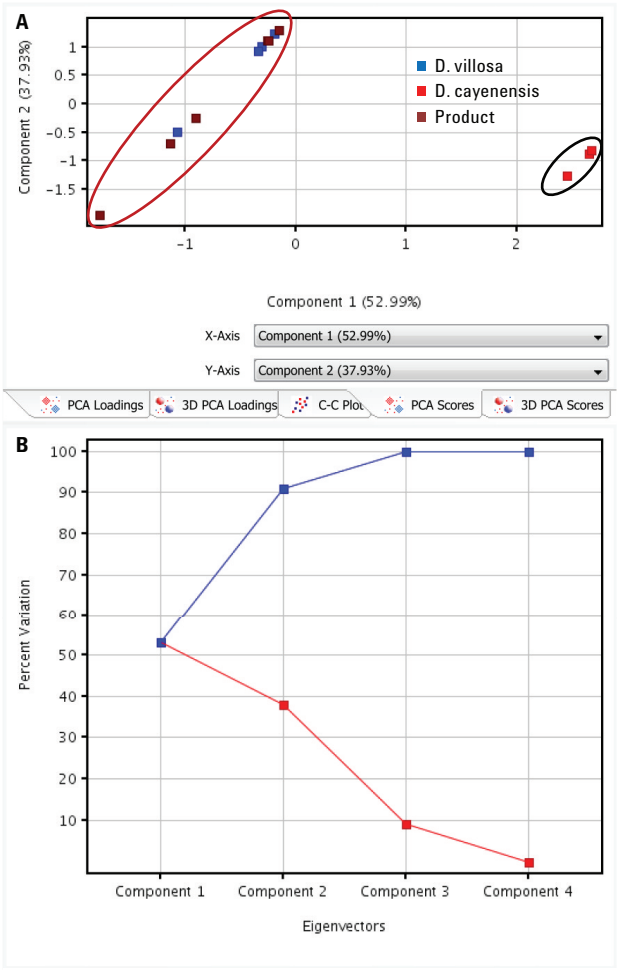


*Figure 2. Agilent Mass Profiler Professional screenshot of A) 2-D PCA showing the first two principal components (PCs) performed on four known D. villosa samples, three known D. cayenensis samples, and five dietary products purported to contain Dioscorea extracts. The PCA used the UHPLC-MS peak area data of the following compounds: parvifloside, huangjiangsu A, pseudoprotodioscin, 26-O-β-D-glucopyranosyl-3β,26-diol-25(R)-furost-5,20(22)-dien-3-O-α-L-rhamnopyranosyl(1→2)-O-β-D-glucopyranoside, 25(R)-dracaenoside G, zingiberensis saponin I, asperin, and gracillin. B) Shows the percent variation explained by each PC, with the blue line showing total variation explained and the red line the variation explained by each PC. Note that 91% of the variation in the data is explained with the first two PCs.*

## Conclusion

A rapid and efficient UHPLC/QTOF MS method has been successfully developed for the characterization of steroidal saponins in the extracts from *Dioscorea* species, using the Agilent 1290 Infinity LC System and an Agilent 6500 Series Accurate-Mass Q-TOF LC/MS. As a major advantage to many previous methods, it does not require long and laborious purification of compounds from crude extracts. The results of this study also suggest that this method can be used to differentiate two *Dioscorea* species and dietary supplements with high sensitivity and reproducibility. This method has the potential to enhance understanding of the biological activity of *Dioscorea* roots and their pharmacological benefits, and to facilitate herbal identification and quality control.

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