

# Optimization of UPLC<sup>®</sup>-Unique<sup>®</sup> TOFMS for the Analysis of Legume Phenolics

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## 1. Introduction

Legumes (Fabaceae) are one of the most economically important crop families in the world. They are planted on about 15% of the world's arable land and provide more than one-third of human protein intake and approximately 35% the world's vegetable oil. In the U.S. alone, over 73 million acres of soybeans (*Glycine max*) and 76 million acres of alfalfa (*Medicago sativa*) were cultivated in 2003 and had estimated values of \$17.5 billion and \$7.5 billion respectively.<sup>1</sup>

Legumes are also a unique source of natural products (or small molecules), many of which have documented antimicrobial and pharmacological properties.<sup>2,3</sup> We are interested in the natural products found in legumes and are utilizing a LECO Unique<sup>®</sup> HT TOFMS coupled to a UPLC (Waters Corp.) to profile, identify, and quantify the natural products in *Medicago truncatula* (barrel medic), *Medicago sativa* (alfalfa), and other legume species. A significant proportion of legume natural products are phenolics which are typically analyzed in the negative-ion ESI mode which has reduced chemical noise and translates to greater S/N ratios. The Unique TOFMS performance is routinely optimized and validated using an Agilent tune mix. However, it was found that the optimized parameters obtained with the Agilent tune mix were not optimal for phenolic analysis. Thus, a standard mix containing four phenolics was generated and utilized to optimize the instrumental parameters.

## 2. Experimental Conditions

### Tune Solution

Agilent tune mix (electrospray calibrant solution, cat#: G2421A) was obtained from Agilent. Four phenolics including quercetin (cat#: Q0125), rutin (cat#: 78095), naringin (cat# N1376), esculin (cat#: E8250) were obtained from Sigma and used directly. The LECO Unique TOFMS was first tuned by direct infusion of the Agilent tune mix and then optimized with the phenolics standards mix.

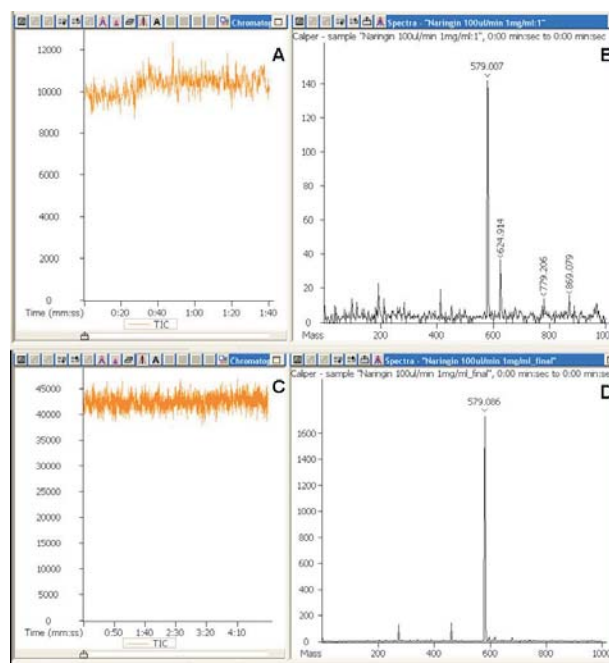
### Instrumentation

LC: Waters ACQUITY UPLC<sup>®</sup>  
Column: ACQUITY UPLC BEH C18, 1.0 mm x 100 mm, 1.7  $\mu$ m  
Flow rate: 100  $\mu$ l/min  
Injection volume: 1  $\mu$ l  
Mobile phase A: 0.1% formic acid in water  
Mobile phase B: Acetonitrile  
Gradient: 95% A to 30% A over 30 minutes

MS: LECO Unique<sup>®</sup> HT TOFMS  
Source: High Flow ESI Source  
Voltage: (-) 4500 v  
Desolvation Temp.: 200°C  
Nebulizer Pressure: 200 kPa  
Interface Temp.: 100°C  
Acquisition Rate: 3.13 spectra/second

## 3. Results and Discussion

The Agilent tune mix consists of betaine and a mixture of substituted phosphazenes of different molecular weights, which produce singly charged ions by ESI. While this tune mixture is widely used to validate and optimize the performance of mass spectrometers in both positive and negative modes, it was found that the optimized parameters obtained using this calibrant solution were not optimal for the analysis of phenolics. Thus, a standard mixture of four phenolics was tested and used to tune the instrument for optimal sensitivity. The optimization was performed mainly in the nozzle-skimmer-quad RF region to maximize the ion generation and transmission into the TOF region. The parameters optimized included desolvation, electrospray, nozzle, skimmer, quad RF, and Einzel vertical deflect voltages. Representative TOFMS spectra acquired before and after optimization are shown in Figure 1. After the optimization, the signal intensity increased dramatically. For the phenolic compound naringin, the signal intensity for the molecular ion ( $m/z$  579) increased by more than tenfold as shown in Figure 1.



**Figure 1. Optimization of the Unique TOFMS for phenolics analysis. TIC of naringin and its TOF-MS spectra acquired before (A, B) and after optimization (C, D) by direct infusion rate of 1.0 mg/ml Naringin in methanol at 100  $\mu$ l/min.**

**Parameters:**  
Desolvation (cc/min): 7023  
Desolvation Temp (°C): 150  
Electrospray (V): -4500  
Nozzle (V): -160  
Skimmer (V): -80  
Quad RF (V): 260  
Einzel vertical deflect (V): 0.6

After optimization using direct infusion, the Unique TOFMS was coupled to a UPLC (Waters) and 1  $\mu\text{l}$  of the phenolic standard mix (containing 10 pmol/ $\mu\text{l}$  of quercetin, rutin, naringin and esculin) was injected and resolved on a reversed-phase column (2.1 mm x 50 mm, ACQUITY UPLC™ BEH C18 1.7  $\mu\text{m}$ , flowrate: 100  $\mu\text{l}/\text{min}$ ). Figure 2 shows the extracted ion chromatogram of the four phenolics. The peak widths were determined to be 5.1 seconds FWHH (full width at half height) for esculin, 5.4 seconds for rutin, 6.8 seconds for naringin, and 7.0 seconds for quercetin. The separation efficiencies expressed in plate number were found to be 7608, 35259, 31163, 50149 respectively, with an average plate number being 31045.

The corresponding TOFMS spectra are shown in Figure 2 and Figure 3 (see back page). For all phenolics except esculin, the base peaks were found to be the molecular ions  $[\text{M}-\text{H}]^-$ . For esculin, the base peak was the aglycon ion or the  $\text{Y}_0^-$ , but the molecular ion ( $m/z$ : 339) was still observed.<sup>4</sup>  $\text{Y}_0^-$  ions were also observed for naringin ( $m/z$ : 271), rutin ( $m/z$ : 301) and quercetin ( $m/z$ : 301 which is also the molecular ion). Crossing-ring cleavage of the hexose to yield  $^{0,2}\text{X}_0^-$  was also observed for naringin.<sup>4</sup> For quercetin, neutral loss of  $\text{CH}_2\text{O}$  was observed ( $m/z$ : 271) and fragment containing the ring X ( $m/z$ : 179) was also observed.<sup>5</sup> The spectra show that under the optimized condition, both the molecular weight and the structural information were obtained, leading to the unambiguous identification of the phenolics. These phenolics are now routinely used for external calibration of the Unique TOFMS. Coupled to UPLC, the Unique TOFMS is a valuable tool in high-throughput metabolomics of plant secondary metabolites.

#### 4. Conclusion

LECO's Unique TOFMS was coupled to a UPLC for phenolics analysis and optimized MS method determined. The MS sensitivity increased significantly following the optimization of voltages related to ion generation and transmission through the RF quadrupole. Tests with four phenolics standards demonstrated that the UPLC-Unique TOF-MS system can be employed in for the analysis of legume secondary metabolites.

#### 5. References

- <sup>1</sup>USDA-NASS (2004) USDA-NASS Agricultural Statistics 2002. [http://www.usda.gov/nass/pubs/agr04/acro04.htm.]
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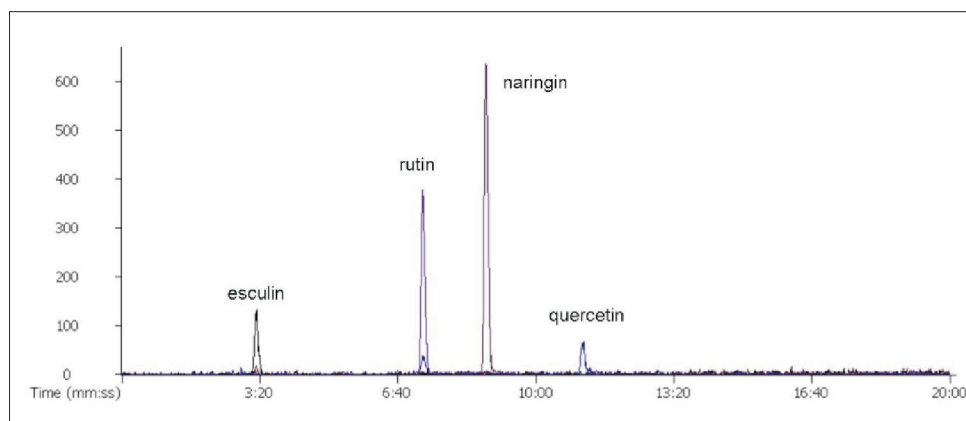


Figure 2. Extracted ion chromatogram of the phenolic standard mix



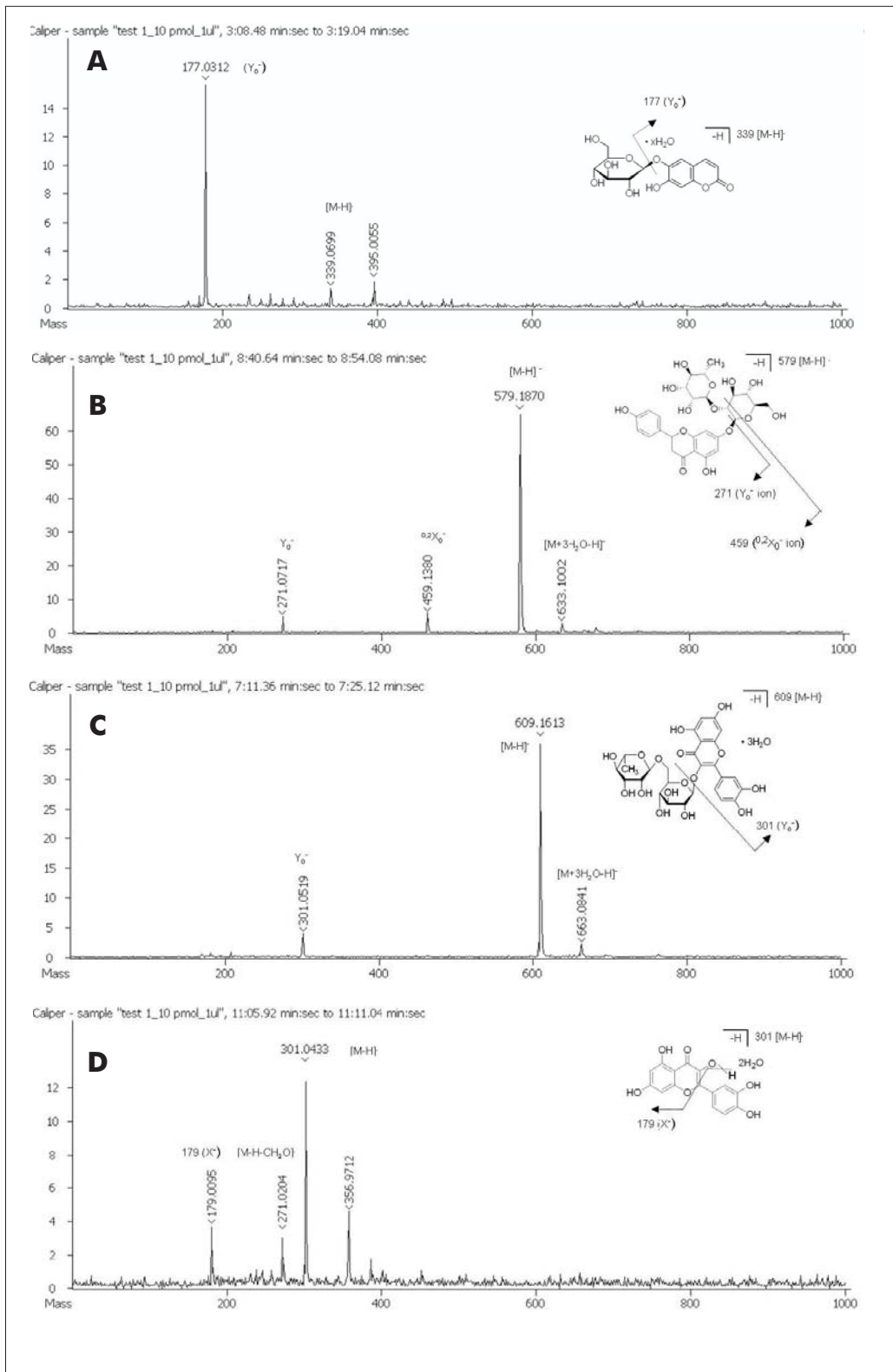


Figure 3. TOFMS spectrum eluted from UPLC: A) esculin, B) naringin, C) rutin, and D) quercetin.