SMALL SCALE NATURAL PRODUCT ISOLATION USING UPLC™ WITH MASS-DIRECTED PURIFICATION™

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INTRODUCTION

Throughout history, the benefits of adopting natural remedies for a variety of health-related maladies has been attractive, as the plants from which these therapeutics are obtained are often readily available.[1] Plant flavonoids, with their diverse biological properties, can be used as antiallergenics, antivirals, and anti-inflammatories, or possess heart-protective vasodilating properties.[2, 3, 4] To fully understand the impact of specific flavonoids in living systems, material needed for experimental studies must often be isolated from very complex matrices. Although traditional isolations have usually been accomplished using extraction followed by chromatography with UV detection, this long and arduous process is riddled with ambiguity due to the lack of specificity in target compound identification. Tedious workup of fractions collected by UV-only detection requires time and resources. Mass detection, with its high specificity and sensitivity, readily discriminates the product of interest from complicated sample mixtures, thereby reducing downstream sample processing time. With improvements in column stationary phases and analytical instrumentation, good separations are more easily realized. The fluidically-optimized flow path of the UPLC, combined with a specially-designed low dispersion fraction collector, enable the mass-directed isolation of sharp, narrow product peaks. In this study, we illustrate the utility of the ACQUITY UPLC H-Class and Waters Fraction Manager-Analytical (WFM-A) Systems for the analysis and isolation of a natural product at the small scale. Fast, targeted isolation increases purification efficiency by reducing unnecessary sample handling while generating just enough product for future experiments.

INSTRUMENTATION

UPLC System controlled by MassLynx™ Software with FractionLynx™ Application Manager: ACQUITY™ UPLC™ HClass, ACQUITY Sample Manager, ACQUITY PDA Detector, ACQUITY QDa™ Detector, ACQUITY Isocratic Solvent Manager (ISM), Waters™ Fraction Manager-Analytical (WFM-A).

EXPERIMENTAL CONDITIONS

LC Conditions

Column and flow rate: ACQUITY UPLC BEH C_{18}, 2.1x50 mm, 1.7 µm, 130Å, Part number 186002350; 0.5 mL/min

Mobile phase A: 0.1% formic acid (FA) in water

Mobile phase B: 0.1% formic acid in acetonitrile (ACN)

Column temperature: 30˚C

Makeup solvent: 9:1 methanol:water, 0.01% formic acid

ISM flow rate: 0.4 mL/min

Wavelength: 251 nm

Gradients and injection volumes: as noted in figures

Samples

4g Egyptian licorice root was extracted overnight in 70% methanol/30% water with shaking. The supernatant was filtered through a 0.45µm syringe filter.

Glycyrrhizic acid standard (Sigma 50531) was dissolved in dimethylsulfoxide, filtered, and diluted to a final concentration of 0.97 mg/mL.

QDa Conditions

MS Scan, 100-1250 m/z, ES+ and ES-, Centroid

Sampling frequency: 2 Hz

Cone voltage: 10 V

Probe Temperature: 500˚C

ESI Capillary Voltage: Positive Mode, 1.5; Negative Mode, 0.8

RESULTS AND DISCUSSION

Glycyrrhiza glabra, or licorice root, has been used as a sweetener and flavor enhancer in food products, as well as a therapeutic, for thousands of years in many parts of the world.[5, 6] The isolation of target compounds from complex matrices is challenging, especially if non-specific detection methods, such as UV, are used for analysis. In this study, we isolated glycyrrhizic acid (Figure 1), the main component of licorice root[7], as well as three closely-eluting impurities, using UPLC with mass-directed purification. The specificity of mass analysis, combined with auxiliary UV detection, provided a more comprehensive profile of the crude extract and simplified target compound isolation. Figure 2 shows the UV profile of the crude licorice extract and the glycyrrhizin standard, as well as the extracted ions for glycyrrhizic acid.

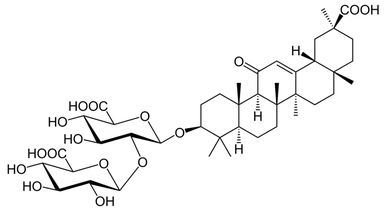

Figure 1. Structure of glycyrrhizic acid, monoisotopic mass 822.4

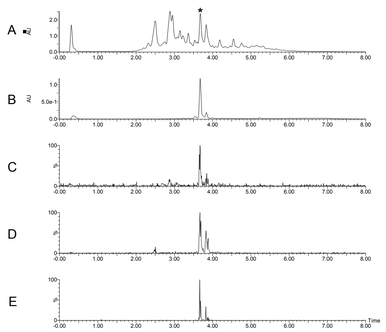

*Figure 2. *Target peak identification in the crude licorice extract, retention time 3.67 min. (A) Total absorbance chromatogram; (B) Glycyrrhizic acid standard, 251 nm; (C) $[M+H]^+$ m/z = 823.4; (D) $[M+Na]^+$ m/z = 845.4; (E) $[M-H]^-$ m/z = 821.4; 5-95%B in 5 min; Inj. Vol. 5 µL.*

Improving the resolution between the peak of interest and the closely-eluting impurities in the crude extract usually ensures better compound purity for the compounds which will be isolated. Although focusing the gradient improved the resolution only marginally, changing the composition of the mobile phase and reducing the gradient range enhanced the separation significantly (Figure 3).

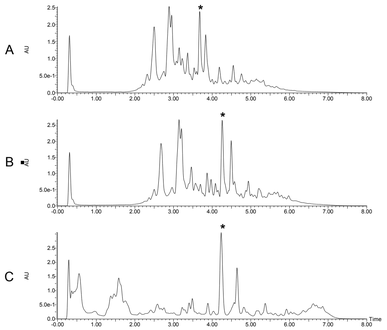

*Figure 3. Chromatographic method development for the *target peak in the crude licorice extract; 251 nm; Inj. Vol. 5 µL. Mobile phase A = 0.1% FA in water (A) 5-95%B in 5 min; mobile phase B = 0.1%FA in ACN; (B) 5-95%B in 5 min; mobile phase B = 0.1%FA in 3:1 ACN:methanol (C) 25-70%B in 5 min; mobile phase B = 0.1%FA in 3:1 ACN:methanol.*

Optimal resolution for the glycyrrhizic acid (4.2 min) was obtained with the 25-70%B gradient in 5 minutes with mobile phase B as 3:1 acetonitrile:methanol with 0.1% formic acid (Figure 3C). Sixty injections with collection of the glycyrrhizic acid (Figure 4, peak 1) , as well as three other impurities (peaks 2, 3, and 4) were completed. A minor impurity eluting before the glycyrrhizic acid was collected, but later discarded without further analysis.

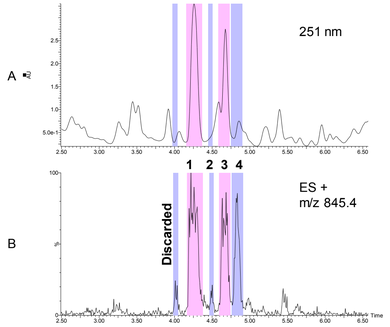

Figure 4. Mass-directed isolation of glycyrrhizic acid (peak 1) from the crude licorice extract; Inj. Vol. 10 µL. (A) UV chromatogram at 251 nm. (B) Extracted chromatogram for the $[M+Na]^+$ m/z = 845.4 ion. 25-70%B in 5 min; Mobile phase A = 0.1% FA in water; mobile phase B = 0.1%FA in 3:1 ACN:methanol.

The collected fractions were pooled by peak number and then analyzed with the 25-70%B gradient. Peak 1, the glycyrrhizic acid isolated from the crude extract, had a retention time of 4.2 minutes. The glycyrrhizin standard eluted at the same retention time (Figure 5), suggesting that the isolation of the target compound was successful.

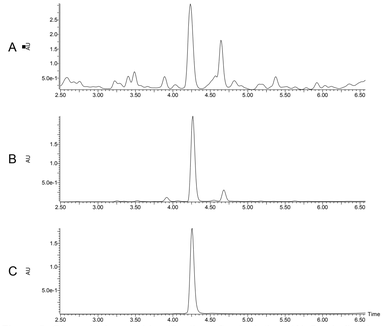

Figure 5. Glycyrrhizic acid identification after isolation. (A) Crude licorice extract, Inj. Vol. 5 µL; (B) Glycyrrhizic acid standard, Inj. Vol. 5 µL; (C) Peak 1 isolate, Inj. Vol. 10 µL; 251 nm.

Fraction analysis on all of the collected peaks showed very good purity by UV at 251 nm (Figure 6).

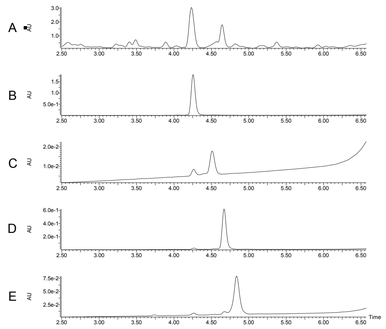

Figure 6. Fraction analysis (A) Crude licorice extract, Inj. Vol. 5 µL; (B) Peak 1 isolate, pool vol. 3.5 mL (C) Peak 2 isolate; pool vol. 1 mL (D) Peak 3 isolate; pool vol. 2 mL (E) Peak 4 isolate; pool vol. 1.5 mL. Isolate Inj. Vol. 10 µL.

Mass and spectral analysis on the isolated glycyrrhizic acid (Peak 1) in both positive and negative modes showed the expected $[M-H]^-$ at m/z 821, in addition to two other ions, m/z 647 and 453, in positive mode (Figure 7). The 647 and 453 ions are most likely fragmentation peaks formed by the neutral loss of two glucuronic acid residues from the $[M+H]^+$ at m/z 823.[7] Similarly, mass and spectral analysis on the glycyrrhizic acid standard produced exactly the same ions in both positive and negative modes. This analogous result suggests the successful isolation of glycyrrhizic acid from the crude licorice extract using mass-directed purification.

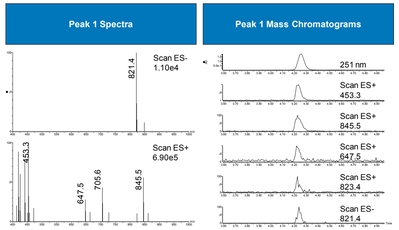

Figure 7. Spectral and mass analysis for peak 1, glycyrrhizic acid, which was isolated from licorice extract.

CONCLUSIONS

- **Natural product isolation is effectively accomplished using UPLC with mass-directed purification for unambiguous target peak identification and collection.**
- **The specially-designed, low-dispersion WFMA simplifies the isolation of narrow, closely-eluting UPLC peaks with fast valve switching and movement between vessels, enabling the collection of target compounds from limited sample sources.**
- **Mass-directed purification at the small scale reduces the time required for post-isolation target compound processing by eliminating extraneous fractions from the workflow.**
- **UPLC with mass-directed purification reduces the time needed to isolate enough compound for ensuing experimental studies when only a small amount of product is required.**
- **Small scale product isolation saves sample, time, and resources, which promotes efficiency in the purification process.**

References

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