Sensitive ESI-LC/MS/MS Analysis of Dansyl Derivatized Phytoestrogens on an Agilent ZORBAX RRHT Eclipse Plus C18 1.8 µm Column

Application Note

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Abstract

Phytoestrogens are naturally occurring nonsteroidal plant compounds, commonly found in the human diet. While their mechanism of action is not fully understood, animal studies have made compelling arguments for their potential treatment and prevention of hormone-dependent diseases, such as cancer, menopausal symptoms, cardiovascular disease and osteoporosis. In this work, an Agilent ZORBAX RRHT Eclipse Plus C18 1.8 µm column is used to analyze a group of dansyl derivatized phytoestrogens. This ammonium acetate/acetonitrile gradient is coupled with ESI-LC/MS/MS detection for a more sensitive analysis, as compared to previous work with APCI detection. Calibration curves for each of the compounds show strong linear correlations, with precise replicate injections, and signal-to-noise >10 for the lowest calibrator.

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Introduction

Phytoestrogens are a diverse group of plant-derived polyphenolic compounds with structural similarities to estrogens, and the potential to interact with estrogen receptors. Because of their possible health effects, there is considerable interest in methods for the analysis of phytoestrogens in various matrices, including biological, food and environmental samples. Atmospheric pressure ionization (API) LC/MS/MS analysis of unmodified phytoestrogens is possible with either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) sources. ESI may be the preferred ionization mode for the analysis of unmodified phytoestrogens, due to somewhat better sensitivity and precision [1]. EI-GC/MS/MS analysis of phytoestrogens as their TMS derivatives has also been described [2].

Several papers on the LC/MS/MS analysis of human estrogens in biological samples describe improved sensitivity by analyzing the estrogens as their dansyl derivatives, in positive ESI mode [3-6]. Improvements in sensitivity of up to two orders of magnitude were reported. This led us to employ the same approach for the analysis of a group of phytoestrogens, as described in this application note. As is the case with human estrogens, sensitivity was significantly enhanced by dansyl derivatization.

Experimental

An Agilent 1200 Series Rapid Resolution LC (RRLC) system with an Agilent 6410 Triple Quadrupole Mass Spectrometer (Triple Quad) was used for this work:

- G1312B Binary Pump SL with mobile phase A: 10 mM ammonium acetate, and B: acetonitrile. Flow Rate was 0.5 mL/min, with the gradient shown in Table 1.
- G1367C Automatic Liquid Sampler (ALS) SL. Injection volume was 2.0 µL with vial well bottom sensing on, and 5 s flushport time.
- G1316B Thermostatted Column Compartment (TCC) SL with temperature set to 40 °C.
- G6410A-2K Triple Quad Mass Spectrometer with MS Source: electrospray AP-ESI, drying gas temperature and flow: 350 °C, 10 L/min, nebulizer gas pressure: 40 psi, capillary voltage: 4000 V, in MRM mode with transitions found in Table 2.
- MassHunter versions B.02.01, B.02.01, B.02.00 and B.04.00 were used for method optimization, data acquisition, qualitative and quantitative analyses respectively.

Table 1. Gradient Program

Time (min)	0:00	3:00	9:00	10:00	13:00
% B	65	65	95	65	65

The method used an Agilent ZORBAX RRHT Eclipse Plus C18, 2.1 mm × 50 mm, 1.8 µm column (Agilent p/n 959764-902).

The structures of the studied compounds and an illustration of the general derivatization scheme using daidzein as an example are shown in Figure 1. Calibrators with final concentrations of 0, 1, 5, 10, 50, 100, and 500 ng/mL of each target phytoestrogen, and containing 100 ng/mL of genestein-D_4 (ISTD) were prepared from methanolic standards in glass screw-capped vials, and evaporated to dryness under nitrogen. One hundred microliters of dansyl chloride (1 mg/mL in acetonitrile, prepared fresh daily) and 100 µL of 10 mM sodium bicarbonate in water were added to each vial, and the vials were capped and heated at 60 °C for 10 minutes. After cooling, the contents of the vials were transferred to screw-capped autosampler vials. Subsequently, 2 µL from each vial were injected in duplicate, resulting in injection of amounts corresponding to 2 – 1000 pg of each underivatized phytoestrogen during calibration.

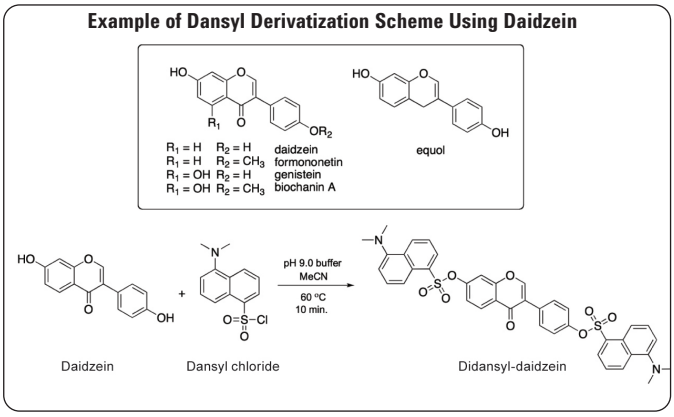

Figure 1. Structures of the compounds of interest.

MS/MS method development was accomplished by injecting approximately 10 ng of individual derivatized phytoestrogens in MS2 Scan mode, using the HPLC column and 80 – 90% B mobile phase. A rapid column separation, rather than flow injection, was employed in order to separate the dansylation reagent and byproducts from the target compounds. MS2 Scan mode was set to scan over a relatively narrow mass range, for example *m/z* 400 – 600 for dansyl-formononetin, with expected precursor ion mass 502, to reduce reagent background signal. Once the expected precursor ion was verified in MS2 Scan mode, MassHunter Optimizer was used (with rapid column separation) to systematically identify fragmentor voltages, product ions, and collision energies. All compounds fragmented to the product ion 170.1, which corresponds to the dimethylaminonaphthalene portion of the dan-

syl group that remains after cleavage of the naphthyl-sulfonyl C-S bond. In addition, three of six phytoestrogens fragmented to significant second product ions, which were used as qualifier ions. In the MRM acquisition method, two MassHunter time segments were employed, with the mobile phase diverted to waste and the data not stored for one minute, followed by acquisition with data stored and a delta EMV of 200 V, and dwell times set at 100 ms. The optimized MassHunter acquisition scan segments are shown in Table 2.

Phytoestrogen standards were purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ), with the internal standard from Cambridge Isotope Laboratories (Andover, MA), and derivatizing agent from Sigma-Aldrich (St. Loius, MO).

Additionally, acetonitrile, ammonium acetate and sodium bicarbonate were also purchased from Sigma-Aldrich (St. Loius, MO). Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Results and Discussion

The phytoestrogen dansyl derivatives were well resolved and gave excellent peak shapes. As expected, the MRMs for genistein and the internal standard genistein-D_4 overlapped, as illustrated in Figure 2, which shows the extracted MRMs in MassHunter Qualitative Analysis for the 10 ng/mL calibrator.

Table 2. MassHunter Acquisition Scan Segments

Phytoestrogen	MW	Number dansyl groups	RT	Precursor ion	Product ion(s)	Fragmentor (V)	CE (V)
Formononetin	268.3	1	2.7	502.2	170.1	170	32
					269.1	170	28
Biochanin A	284.3	2	7.64	751	170.1	140	32
					517.1	140	16
Daidzein	254.2	2	7.94	721	170.1	226	48
Equol	242.3	2	8.54	709	170.1	220	56
Genistein	270.2	3	10.08	970.3	170.1	210	56
					736.1	210	24
Genistein-D_4	274.2	3	10.07	974.3	170.1	210	56

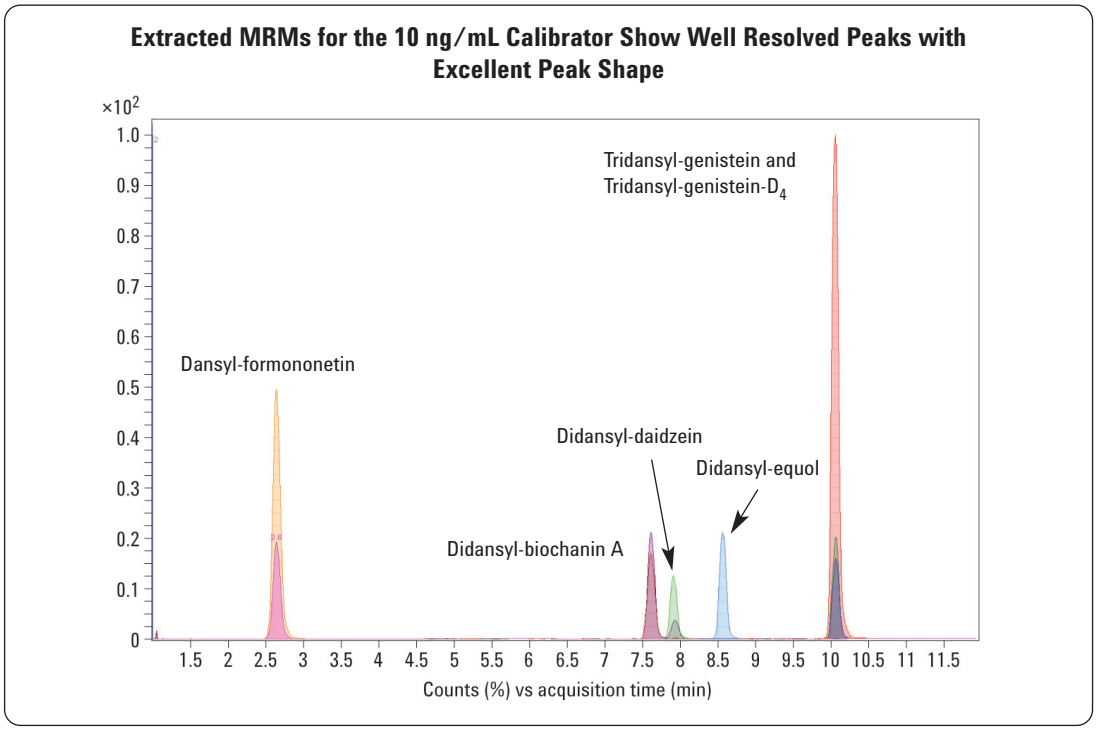

Figure 2. Chromatographic separation of phytoestrogen dansyl derivatives with a 2.1 mm × 50-mm, 1.8-µm Agilent ZORBAX RRHT Eclipse Plus C18 (See Experimental section for detailed method parameters).

The MS/MS Integrator was used for all compounds. The lowest calibrator (1 ng/mL) gave signal-to-noise results of >10 for all target compounds, using either the default RMS noise algorithm, or the alternative peak-to-peak algorithm. Calibration curves for all analytes except equol were linear from 1 – 500 ng/mL, corresponding to injected amounts of 2 – 1000 pg of phytoestrogens prederivatization. Equol calibration was linear from 1 – 100 ng/mL. Duplicate agreement was excellent, as shown in the calibration curve for biochanin-A ($1/x^2$ weighting) in Figure 3. After derivatization, calibrators were stable for at least 24 hours.

Two minor contaminants presenting potential interference problems were present in the internal standard. One small peak with the same retention time and single MRM as didansylated-equol gave an estimated concentration of approximately 0.2 ng/mL equol in the blank. A second peak with a slightly greater retention time than didansylated biochanin-A gave the quantitating ion, but not the qualifier ion for that analyte. Two advanced features of MassHunter Quantitative Analysis were used to resolve this potential interferent. In the Method Editor, Method Setup Task, Retention Time Setup, Peak Selection Criteria for biochanin-A was changed from the default setting of Greatest Response to Close RT. This gave the correct peak identification, even when the interferent peak was larger than the target peak. In the Globals Setup Task in the Method Editor, the Non Reference Window was changed from the default of 200% to 5%, which resulted in only the target peak being integrated. Figure 4 shows the Compounds at a Glance view for the blank and standards for biochanin-A, illustrating the resolution of the potential interferent.

Example Calibration Curve for Biochanin-A is Linear from 1–500 ng/mL (2–1000 pg of phytoestrogens prederivatization), with Excellent Precision using Duplicate Injections

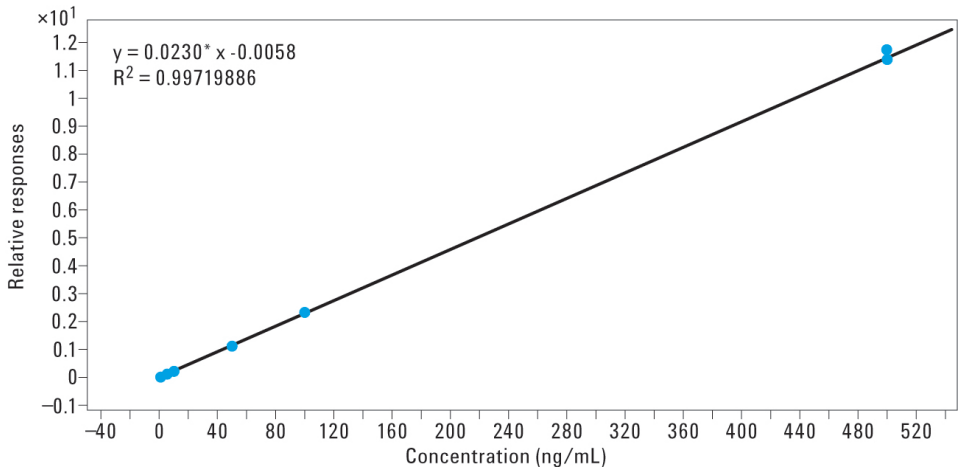

Figure 3. MassHunter-generated calibration curve for biochanin-A ($1/x^2$ weighting).

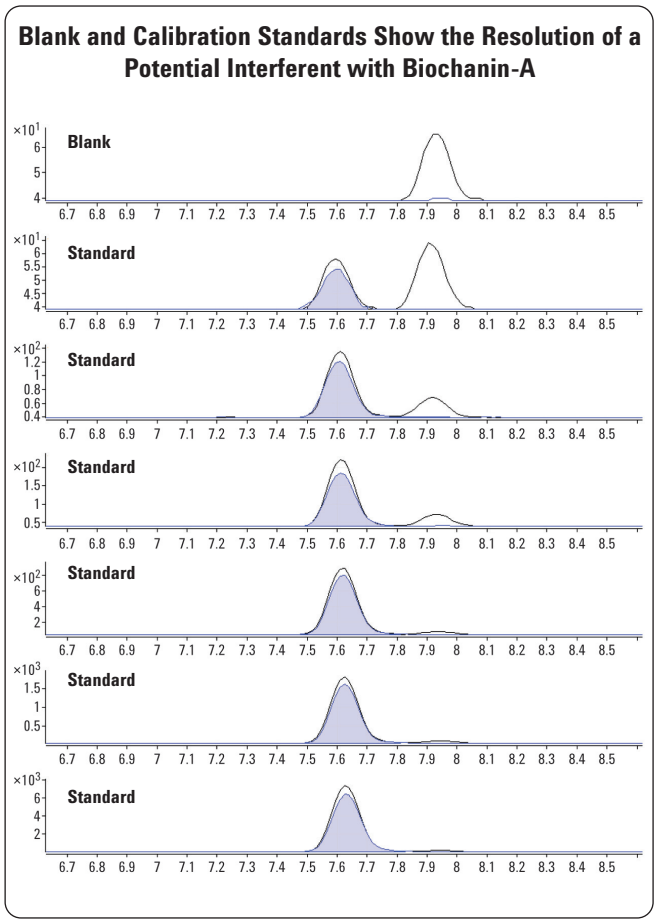

Figure 4. MassHunter's Compounds at a Glance view for the blank and standards for biochanin-A.

Initially, LC/MS/MS method development for a study of phytoestrogen photodegradation began by analyzing the unmodified compounds in APCI mode, but did not achieve the sensitivity needed for the planned investigation. After reviewing the literature on human estrogen analysis, it was decided to evaluate phytoestrogen analysis as the dansyl derivatives. Phytoestrogen dansyl derivatives displayed good chromatographic behavior, and significantly lower detection limits (low ppb) in positive ESI mode than previously achieved when analyzing the unmodified compounds in APCI mode. Insufficient sensitivity for the analysis of unmodified human estrogens in ESI or APCI mode has been reported, and dansyl and other derivatives have been employed to improve sensitivity [7]. Kushnir et al optimized dansylation conditions for estrone and estradiol analysis in serum, and found best recovery at higher pH and dansyl chloride concentrations [8]. Kushnir et al also describe the potential limitations of the use of dansyl derivatives, notably the nonspecific fragmentation to the dimethylaminonaphthalene that forms upon cleavage of the naphthylsulfonyl C-S bond of the dansyl group. The Optimizer results for the dansylated phytoestrogens indicated that some, but not all of the derivatives yield additional product ions with sufficient intensity to use as qualifier ions. As noted by Kushnir, dansyl chloride is highly reactive with hydroxyl and amine groups, so the analyst needs to consider the potential for matrix interferences caused by derivatization. Matrix interferences are a relatively minor consideration for this application, because the samples are relatively clean following solid phase extraction. One other possible drawback of derivatization is the potential for hydrolysis of conjugated phytoestrogens during derivatization, leading to elevated results [9]. Hydrolysis of conjugates is not a concern for these photodegradation studies of unconjugated phytoestrogens, but should be evaluated if this method is applied to more complex biological samples.

Several method modifications may be pursued during further method development. It should be feasible to use a lower internal standard concentration than 100 ng/mL, which would limit the impact of minor contaminants in the internal standard, especially on the quantitation of equol. Also, a higher delta EMV value in the MS/MS acquisition may enable lower limits of quantitation than the current value of 2 pg of prederivatization phytoestrogen injected on-column. Finally, inspection of Figure 2 shows that the MS/MS Acquisition method could easily be divided into multiple time segments, which would allow longer dwell times, and possibly enable lower detection and quantification limits. Use of a Dynamic MRM acquisition would also result in increased dwell times, and could enable lower detection and quantification limits.

API-LC/MS/MS is a very powerful tool for the analysis of many compounds of biological interest. However, one of the main limitations of API is the inability to ionize relatively nonpolar molecules sufficiently to permit subsequent triple quad MS analysis at low concentrations [10]. Derivatization of relatively nonpolar analytes to form compounds that ionize readily expands the scope and sensitivity of API-LC/MS/MS, as demonstrated in this note.

Conclusion

A group of phytoestrogens was successfully analyzed and separated with excellent peak shape using a 1.8-µm Agilent ZORBAX RRHT Eclipse Plus C18 column. This ESI-LC/MS/MS analysis with dansyl derivatization significantly improved sensitivity and precision for all phytoestrogens, as compared to previous APCI methods with unmodified compounds. Replicate injections were very reproducible with calibration curves exhibiting strong linear correlations, and signal-to-noise of the lowest calibrator >10 for all target compounds.

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