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Nota applicativa

Analysis and Purification of Diastereomeric Steroid Saponins from the *Trigonella foenum-graecum* Seed Extract Using SFC/ELSD

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Abstract

This application note demonstrates the first successful separation of a pair of 25 *R/S* spirostanol saponin diastereomers using SFC is demonstrated in this application note. As isomeric separation is often a prerequisite for accurate bioassays in natural product drug discovery, SFC readily lends itself as a complementary chromatographic technique to HPLC, especially for diastereomeric resolution.

Benefits

The structural complexity of steroid saponins presents a daunting challenge in resolving their stereoisomers, especially those 25 *R/S* diastereomers. As a result, bioassays are often performed on the isomeric mixtures, which might lead to skewed results.

Introduction

Biosynthesis enzymes are inherently three-dimensional. As a result, enzymatically-derived natural products are usually structurally diverse, sterically complex, and chiral. Compared to synthetic compounds, natural products contain significantly more rings and chiral centers.[1] For example, steroid saponins are an important class of compounds widely distributed in plant and marine animal kingdoms. They are glycosides consisting of one or more oligosaccharide chains, linked to a steroid aglycone with multiple five- and six-member ring structures. In addition to different oligosaccharides, stereochemistry at C-25 also contributes to their structural variability. Spirostanol saponins, shown in Figure 1, are a subgroup of steroid saponins, and refer to those containing spirostane-type of aglycones.

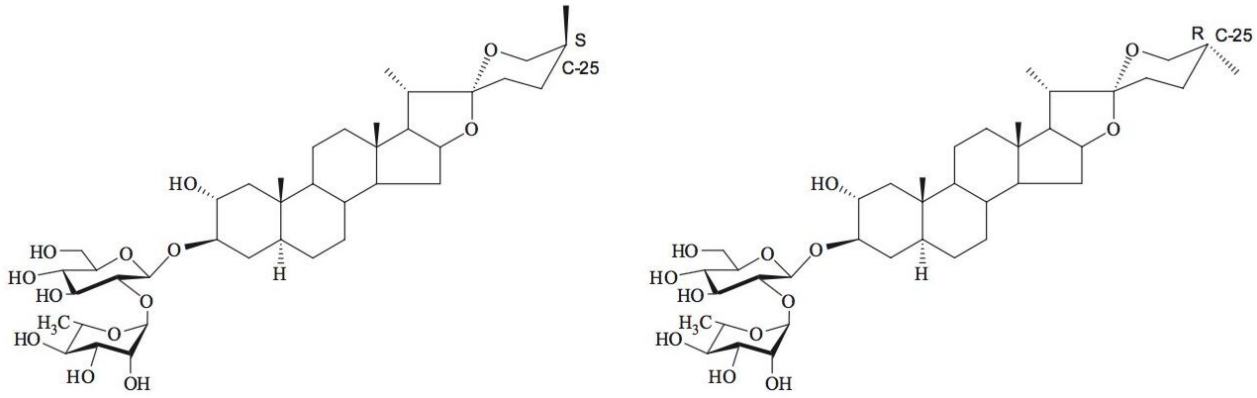

Figure 1. Chemical structures of a spirostanol saponin.

Steroid saponins show many pharmacological activities, including anticancer, cytotoxicity, anti-inflammatory, antioxidant, antiviral, antifungal, and antimicrobial, to name a few. For example, the seeds of *Trigonella foenum-graecum* L. are rich in steroid saponins and have long been used as a remedy for tonic and stomachic purposes in Traditional Chinese Medicine (TCM).[2] There is a substantial research effort to isolate steroid saponins from various plants using TLC and HPLC, followed by structural elucidation and bioassays.[3-6] However, the structural complexity of steroid saponins presents a daunting challenge in resolving their stereoisomers, especially those 25 *R/S* diastereomers. To date, there has not been a successful resolution of 25 *R/S* spirostanol saponin isomers by any chromatographic technique.[7-8] Instead, bioassays were performed on the isomeric mixtures, which might lead to skewed results as optical isomers could possess considerably different bioactivities.

Herein, we report the chromatographic resolution of a pair of 25 *R/S* spirostanol saponin diastereomers from the *Trigonella foenum-graecum* L. seed extract using SFC/ELSD, both analytically and preparatively. The advantages of using SFC for spirostanol saponin analysis and purification are discussed.

Experimental

SFC method conditions

Analytical SFC

Flow rate:	3.5 mL/min
Co-solvent:	Methanol
Back pressure:	120 bar
Temp.:	40 °C
Co-solvent percentage:	33%
Column:	CHIRALPAK IC (4.6 x 150 mm or 250 mm or 400 mm, 5 µm)
ELSD:	N_2 pressure: 35 psi, Drift tube temp.: 60 °C, Nebulizer heater: 85%
Injection vol.:	10 µL

Preparative SFC

Flow rate:	3.5 mL/min
Co-solvent:	Methanol
Back pressure:	120 bar
Temp.:	40 °C
Co-solvent percentage:	33%
Column:	CHIRALPAK IC (4.6 x 150 mm, 250 mm, or 400

	mm, 5 µm)
ELSD:	N_2 pressure: 35 psi, Drift tube temp.: 60 °C, Nebulizer heater: 85%
Injection vol.:	75 µL

Instrumentation:

Analytical method development was performed on a Waters Method Station SFC System controlled by MassLynx Software. The Method Station SFC System consists of the following components: Fluid Delivery Module (FDM), Automated Back Pressure Regulator (ABPR), Alias Autosampler, 10-port Analytical-2-Prep Column Oven, and 2424 Evaporative Light Scattering Detector (ELSD).

All preparative experiments were performed on a Waters Investigator SFC System controlled by ChromScope Software. The Investigator SFC System consists of the following components: FDM, ABPR, Alias Autosampler, 10-port Analytical-2-Prep Column Oven, 2424 ELSD, make-up pump, and six-position Fraction Collection Module. A tee was placed immediately before the ABPR to split the flow to the ELSD and the ABPR at a ratio of approximately 1:20.

Chemicals:

SFC-grade CO_2 was provided by Air Gas (Salem, NH, USA). HPLC-grade methanol was purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA).

Sample preparation:

The seeds of *Trigonella foenum-graecum* L. were purchased from Anguo, Hebei province, China. Extraction and isolation were previously described elsewhere.[9] The sample was white, amorphous powder dissolved in methanol at approximately 1 mg/mL prior to SFC.

Results and Discussion

With very few exceptions, most saponins do not possess chromophores necessary for UV detection. Therefore, ELSD and MS have been coupled to HPLC for detection of saponins.[10-11] In our experiments, we

chose ELSD as the choice of detection to ensure the continuity between analysis and purification. Since ELSD response is highly dependent on the co-solvent percentage,[12] isocratic methods were developed for the separation of the 25 *R/S* spirostanol saponin diastereomers, to accurately assess the isocratic ratio. Figure 2 shows the RPLC/ELSD, and SFC/ELSD chromatograms. No separation was observed with RPLC. Using a 250-mm CHIRALPAK IC column, the resolution of the isomeric pair was 1.76 and the isomeric ratio (peak 1/peak 2) was 0.64.

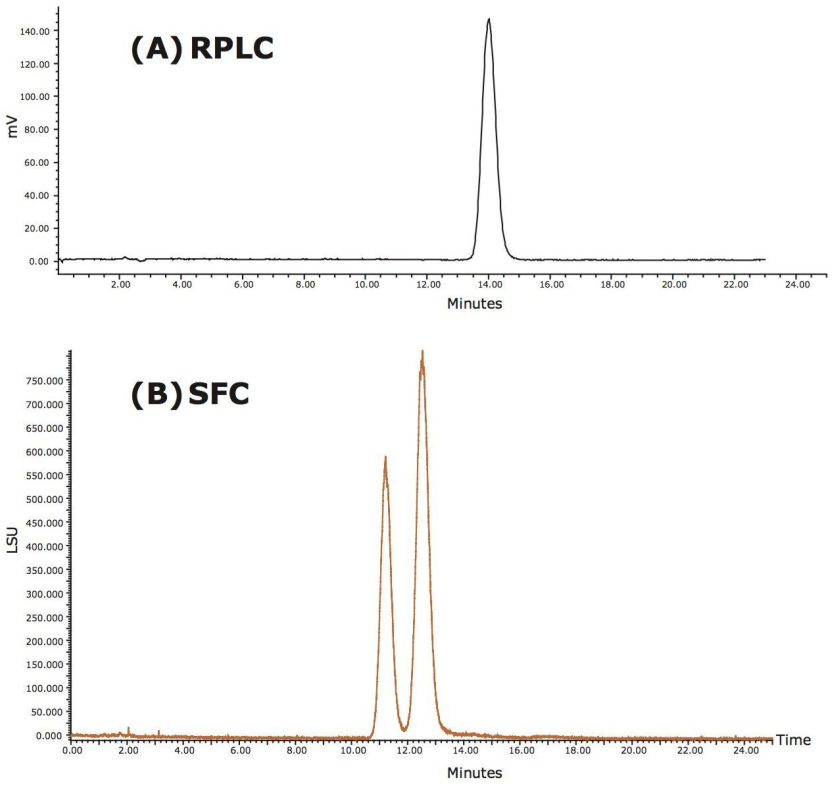

Figure 2. RPLC/ELSD (A) and SFC/ELSD (B) chromatograms of the spirostanol saponin diastereomers. RPLC method conditions were the following: Lichrospher C_{18} (4.6 x 250 mm, 5 µm) column; column heater: 35 °C; mobile phase: 83:17 methanol/water; flow rate: 1.0 mL/min. SFC method conditions are listed in the experimental section.

Resolution has a direct impact on sample loadability in preparative chromatography. A successful preparative chromatography often requires a resolution of 2 or greater. In an effort to further improve the resolution, two IC columns (150 mm and 250 mm) were coupled in tandem, and the resulting chromatograms are shown in Figure 3. The resolution and isomeric ratio for each run were calculated, and are summarized in Table 1.

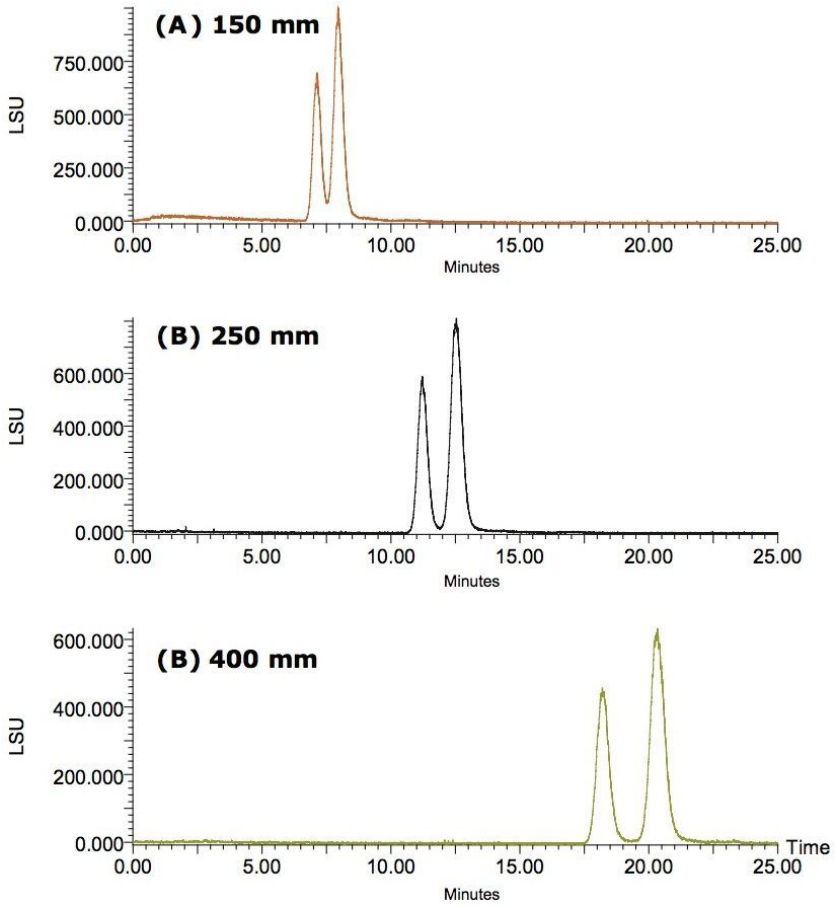

Figure 3. SFC/ELSD chromatograms of the spirostanol saponin diastereomers with different column lengths.

Column length (mm)	t_1 (min)	t_2 (min)	$w_{0.5,1}$	$w_{0.5,2}$	Isomeric ratio (peak1/peak2)	$*R_s$	Increase	Theoretical increase
150	7.14	7.95	0.34	0.39	0.62	1.31	0%	0%
250	11.21	12.54	0.44	0.48	0.64	1.71	31%	29%
400	18.20	20.33	0.55	0.58	0.64	2.23	70%	63%

* $R_s = \frac{2(t_2 - t_1)}{1.7(w_{0.5,1} + w_{0.5,2})}$, where t is the retention time and $w_{0.5}$ is the peak width at half height.

Table 1. Comparison of three SFC runs with different column lengths.

Clearly, as the column length increased, the retention time increased proportionally, while the resolution also increased. The extent of the resolution improvement tracks well with those from theoretical predications, as

described by equations 1 and 2. The isomeric ratios were consistent among all three runs, indicating the column coupling approach taken in our experiments did not cause any peak distortion or sacrifice the chromatographic integrity. Additional peak dispersion, due to increased column length, did not seem to have a noticeable detrimental impact on resolution enhancement. There are two possible factors contributing to this observation. First, high flow rates are often used in SFC, which in turn minimize the analyte residence time on column and reduce the longitudinal dispersion; and secondly, the dispersion resulting from the resistance to mass transfer is also minimal in SFC because of the high diffusivity of liquid CO_2.

$$R_S = \frac{1}{4}\sqrt{N}(\alpha - 1)\frac{k}{k+1} \qquad \textit{Equation 1}$$

$$N = \frac{L}{d_p} \qquad \textit{Equation 2}$$

Increasing column length is one of the most fundamental and facile means to improve chromatographic resolution. However, it is less commonly used in HPLC, due to the limitation posed by the pressure drop across the column, as depicted in equation 3.

$$\Delta P = \frac{250L\eta F}{d_c^2 d_p^2} \qquad \textit{Equation 3}$$

Column coupling has been used for both analytical and preparative SFC,[13-15] when selectivity or retentivity was inadequate to improve resolution. Compared with HPLC, SFC holds a unique advantage because of the low viscosity of liquid CO_2. The viscosity of the liquid CO_2 under typical SFC conditions is 10- to 100-fold lower than those commonly used HPLC solvents; hence, effectively offsetting the ΔP elevation resulting from longer columns.

Next, the optimized method with two IC columns was transferred to the Investigator SFC System for preparative runs. Figure 4 shows a representative SFC/ELSD chromatogram with five stacked injections. With 75-µL injections, baseline resolution was well maintained, allowing for high purity compound collection. Despite the 20-min run time for each injection, with stacked injections, five runs were completed in less than 60 min. Stacked injections is an effective means to improve productivity without compromising chromatographic efficiency. Performed under isocratic conditions, injections are made during the course of chromatography so that the first peak from a subsequent injection elutes off the column adjacent to the last

peak from the preceding injection. The chromatographic space is efficiently populated by peaks from consecutive injections, resulting in noticeable savings of both time and solvents. Post purification analysis indicated 100% purity for both fractions, and the collections were subject to high resolution mass spectrometry (HRMS) and nuclear magnetic resonance (NMR) analyses (results not shown).

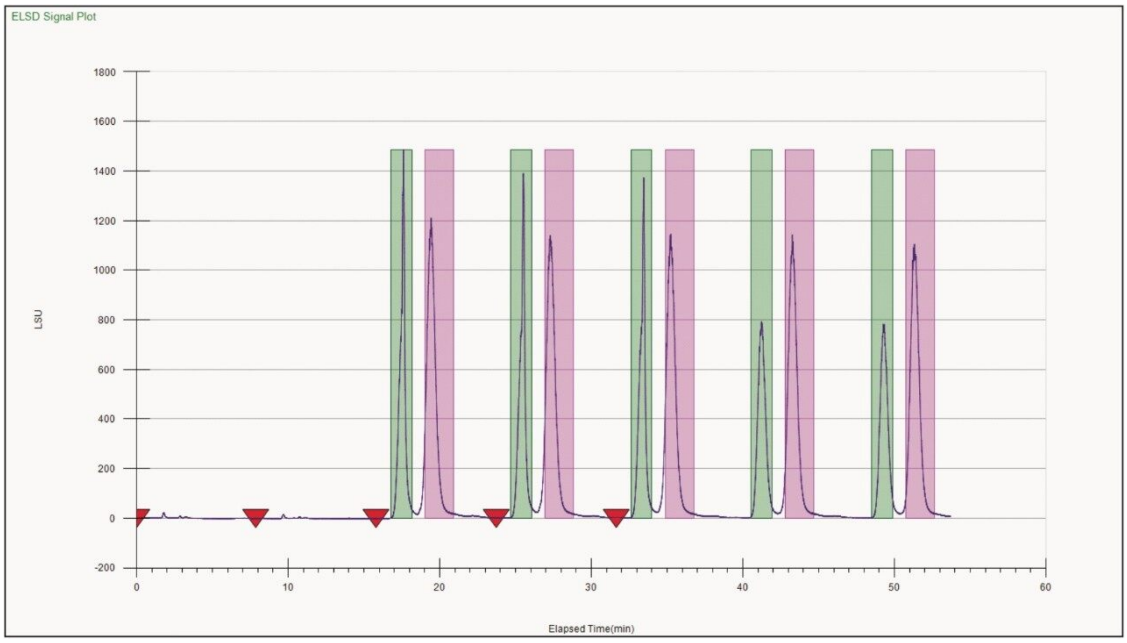

Figure 4. SFC/ELSD chromatogram of the spirostanol saponin diastereomers showing five stacked injections. The shaded area indicates collected fractions.

Conclusion

We have demonstrated, for the first time, the successful chromatographic resolution of a pair of 25 *R/S* spirostanol saponin diastereomers using SFC. Leveraging the low viscosity of liquid CO_2 used in SFC, two columns were coupled in tandem to achieve the desired resolution necessary for preparative runs. In preparative SFC, to circumvent the relative long chromatography time, stacked injections were employed to save both time and solvents. As isomeric separation is often a prerequisite for accurate bioassays in natural product drug discovery, SFC readily lends itself as a complementary chromatographic technique to HPLC, especially for diastereomeric resolution.

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