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Improving the Productivity in Isolating a Naturally Occurring Bioactive Compound Using Supercritical Fluid Extraction and Preparative Supercritical Fluid Chromatography

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Abstract

In this application note, we have demonstrated employing SFE and prep SFC to holistically improve the productivity in isolating a low-polarity, bioactive compound from a complex natural product extract. The SFE alleviated the sample complexity prior to analysis and purification, thereby improving sample loading and reducing solvent use in the ensuing chromatography. The SFE extract also lends itself well for SFC analysis and purification.

Benefits

- SFE alleviates the sample complexity in natural product extracts prior to chromatographic analysis and purification, enabling a more efficient purification downstream.
- SFC offers complementary separation to RPLC. In addition, there is a wide range of column chemistries available in SFC with vastly different separation mechanisms. The combinations of SFC/RPLC and SFC/SFC provide unmatched resolving power to meet the challenges, primarily arising from sample complexity, in natural product isolation.
- Both SFE and SFC reduce the use of organic solvents and provide an easy sample recovery under mild conditions, thereby increasing the overall purification productivity and cost-effectiveness.

Introduction

Natural products are a productive source of leads for new drugs due to their high chemical diversity, biochemical specificity, and many “drug-likeness” molecular properties.¹⁻⁴ A large portion of today’s existing drugs on the market are either directly derived from naturally occurring compounds or inspired by a natural product. In addition, natural products are also used in the forms of food supplements, nutraceuticals, alternative medicines, and as active ingredients in cosmetics.⁵

Isolation and purification of bioactive compounds play an important role in natural product research. The most commonly used process often involves extraction of target compounds from the cellular matrix, pre-purification by various chromatographic techniques including flash chromatography (FC), low pressure liquid chromatography (LPLC), and medium pressure liquid chromatography (MPLC), followed by preparative high pressure liquid chromatography (prep-HPLC).⁶ However, this process is not without its challenges. For example,

conventional extraction methods for natural products include Soxhlet extraction, maceration, percolation, and sonication. These methods are often time- and labor-intensive, consume large amounts of organic solvents, and can lead to the degradation of thermally labile compounds. Furthermore, prep chromatography is largely dominated by reversed-phase liquid chromatography (RPLC), whereby the separation is driven by the differentiating polarity of the analytes. While a generally applicable chromatographic technique for a variety of compound classes, RPLC does not necessarily guarantee an adequate resolution for all analytes, especially for the structural analogs and isomers of similar polarities often found in natural products. As a result, the purification step is perceived by many as a rate-limiting step and a major bottleneck for natural product drug discovery, as well as in the development of differentiated nutraceutical and cosmetic products⁷

To that end, supercritical fluid (SF) based techniques, including supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC), can offer viable additions to the natural product isolation toolbox by leveraging the unique properties of supercritical CO₂: high diffusivity, low viscosity, and superb solvation power. SFE has been successfully applied to the extraction of many bioactive compounds from medicinal plants, including steroids, terpenes, alkaloids, and phenolic compounds.⁶ Preparative SFC has been widely adopted by the pharmaceutical industry for active pharmaceutical ingredient (API) purification. Its applications in natural product isolation, however, remain scarce.⁸

In this application note, we describe a systematic effort to holistically improve the productivity in isolating a naturally occurring terpene derivative with proven anti-cancer bioactivity from a raw plant sample. The process involves an extraction by SFE followed by three different, two-step purification routes, including MPLC+HPLC, MPLC+SFC, and SFC+SFC. The overall productivity and solvent consumption for each purification route are compared.

Experimental

Materials and reagents

HPLC-grade methanol and isopropanol (IPA) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Denatured ethanol (reagent grade) was purchased from Sigma (St. Louis, MO, USA). The fine ground plant material was used as received.

Sample preparation

~~Solvent extraction~~

A total of 0.3 g of ground plant material and 6 mL methanol were placed into a 10 mL test tube. After sonication at 40 °C for 1 hour, the suspension was centrifuged for 5 min. The supernatant was transferred to a clean vial for further analysis.

Supercritical fluid extraction

The extraction experiments were performed on a Waters MV-10 ASFE System controlled by ChromScope Sample Prep Software. A total of 3 g of ground plant material was weighed into a 5 mL extraction vessel. The extraction was performed for 60 minutes with 8 mL/min CO₂. The effluent was carried into a 100 mL collection vessel with a makeup flow of 1 mL/min of methanol/ isopropanol/hexane (1:1:1).

Chromatography

Analytical LC-MS experiments were performed on a Waters ACQUITY UPLC H-Class System/SQ Detector 2 and a Waters AutoPurification LC System. The analytical UPC²-MS experiments were performed on a Waters ACQUITY UPC²-MS System. All systems were controlled by MassLynx software. The MS-directed SFC preparative experiments were performed on a Waters Prep 100q SFC MS-Directed System controlled by MassLynx/FractionLynx Software. All UV-directed preparative experiments were performed on a Waters SFC 80 Preparative System controlled by ChromScope software. Detailed experimental parameters are summarized in Tables 1-3.

	Figure 2A		Figure 2B		Figure 6A	
Instrument	ACQUITY UPLC H-Class System/SQD2 MS		AutoPurification LC MS System		ACQUITY UPLC H-Class System/SQD2 MS	
Flow rate (mL/min)	0.60		1.46		0.75	
Mobile phase A	Water		Water		Water	
Mobile phase B	Methanol		Methanol		Methanol	
Backpressure (psi)	N/A		N/A		N/A	
MS detection	ESI+		ESI+		ESI+	
Column	ACQUITY HSS T3 (1.8 μm, 3.0 x 150 mm)		Atlantis T3 (5 μm, 4.6 x 150 mm)		ACQUITY BEH C ₁₈ (1.7 μm, 2.1 x 50 mm)	
Temperature (°C)	60		Ambient		60	
Injection volume (μL)	1		Varying		0.5	
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	92	0	88	0	80
	5	96	3.08	88	4	80
	5.25	92	8.21	94		
	6	92	8.61	100		
				9.22	88	
			20.90	88		

Table 1. Key experimental parameters for analytical LC.

	Figure 1	Figure 3A	Figure 4A	Figure 4B	Figure 6B					
Instrument	ACQUITY UPC ² System/TQD MS									
Flow rate (mL/min)	1.5									
Backpressure (psi)	1740									
MS Detection	APCI+									
Temperature (°C)	45									
Injection volume (μL)	1									
Mobile phase A	CO ₂									
Mobile phase B	Methanol	Isopropanol	Isopropanol	Isopropanol	Isopropanol					
Column	ACQUITY UPC ² 2-EP (1.7 μm, 3.0 x 100 mm)	ACQUITY UPC ² 2-EP (1.7 μm, 3.0 x 100 mm)	ACQUITY UPC ² 2-EP (1.7 μm, 3.0 x 100 mm)	GreenSep Nitro (1.8 μm, 3.0 x 100 mm)	ACQUITY UPC ² 2-EP (1.7 μm, 3.0 x 100 mm)					
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	5	0	5	0	5	0	20	0	5
	4.5	45	2.50	25	2.50	25	2.5	50	2	25
	5	45	2.75	40	2.75	40	3	50	2.75	40
	5.25	5	3.25	40	3.25	40	3.25	20	3.25	40
	6	5	3.50	5	3.50	5	4	20	3.50	5
		4	5	4	5			4	5	

Table 2. Key experimental parameters for UPC²

	Figure 3B	Figure 5A	Figure 5B			
Instrument	Prep 100q SFC MS-Directed System	SFC 80 Preparative System	SFC 80 Preparative System			
Flow rate (mL/min)	80	80	80			
Mobile phase A	CO ₂	CO ₂	CO ₂			
Mobile phase B	Isopropanol	Isopropanol	Ethanol			
Backpressure (psi)	1740	1740	1740			
Column	Viridis Silica 2-EP (5 μm, 19 x 150 mm)	Viridis Silica 2-EP (5 μm, 19 x 150 mm)	Nitro (5 μm, 21 x 150 mm)			
Temperature (°C)	40	40	40			
Sample diluent	Isopropanol	Isopropanol	Ethanol			
Injection volume (mL)	0.6	3	1			
Collection trigger	MS	UV	UV			
Gradient	Time (min)	%B	Time (min)	%B	Time (min)	%B
	0	5	0	5	0	8
	1	5	5	5	9	8
	6.5	9	7	30		
	7	9	10	30		
	7.25	5	11	5		
8	5	12	5			

Table 3. Key experimental parameters for preparative chromatography.

Results and Discussion

Target compound extraction using SFE

Any solid-liquid extraction process, such as solvent extraction and SFE, is predominantly a solubility driven process. The process involves diffusion of the extracting solvent into the matrix, solubilization of the target analytes in the extracting solvent, diffusion of the target analytes in the extraction solvent, and transport of the extracted analytes into a collection vessel.⁶ Conventional polar extraction solvents, such as alcohols, often produce extracts comprised of mixtures of many polar and non-polar compounds. Supercritical CO₂, on the other

hand, is a highly lipophilic solvent. As a result, only relatively non-polar compounds are typically extracted by SFE using neat CO₂. In the current study, the target compound is a terpene derivative with a nominal mass of 390.28 Da and a LogP of 3.0. The low molecular weight and the relatively low polarity make it an ideal candidate for extraction by SFE.

Figure 1 shows the UPC²-MS chromatograms of two extracts obtained by SFE (Figure 1A) and methanol extraction (Figure 1B) using a BEH 2-EP column. Since 2-EP is a polar stationary phase, the elution order of the compounds generally tracks their polarities; the later the elution, the more polar the compounds. While both extracts contain similar amount of the target compound, it is evident that SFE yielded a much simpler extract compared to methanol extraction. For the SFE extract, the peaks immediately after the target compound (1.20–1.75 min, blue rectangle) are much lower in intensity than those in the methanol extract. The peaks between 1.75–3.50 min (red rectangle) are only present in the methanol extract. Overall, the SFE extract is a much simpler mixture consisting of fewer polar components. The target compound was therefore enriched by SFE prior to chromatography. This makes the SFE extract ideal for large mass loading in prep chromatography and requires relatively low organic co-solvent (mobile phase B) composition to completely elute off the components in the extract; thereby shortening the total run time, reducing the solvent consumption, and increasing purification productivity. Detailed prep SFC experiments are described in a later section.

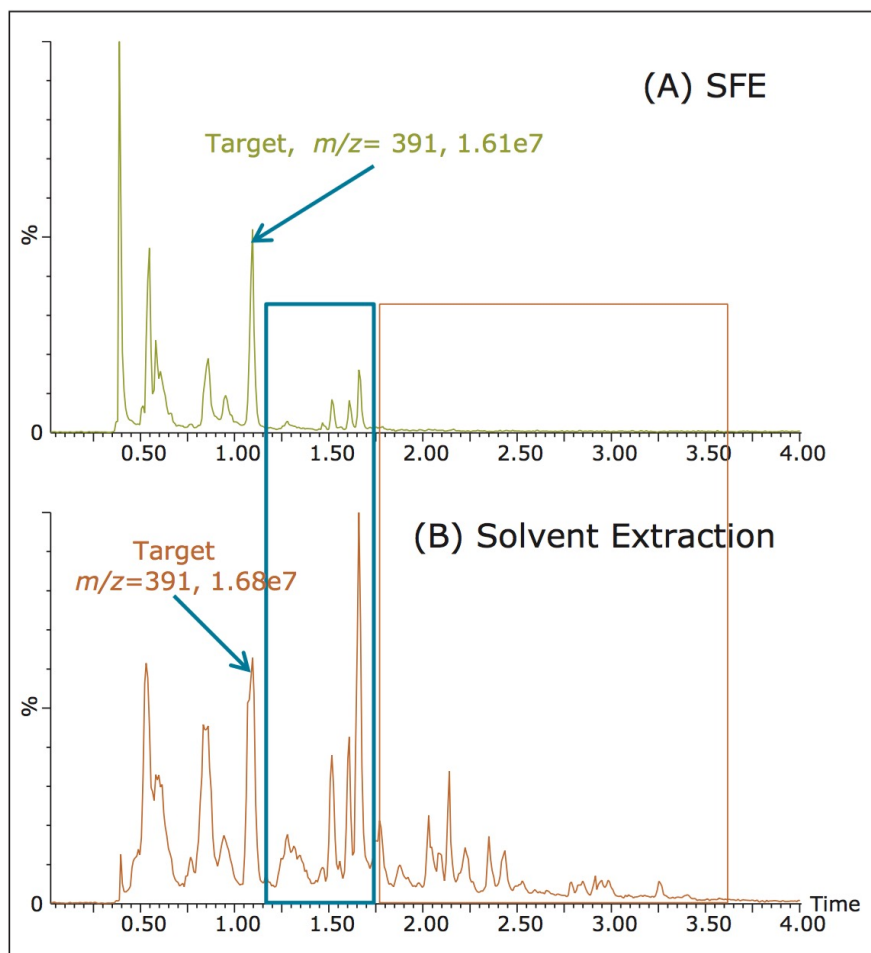


Figure 1. UPC²-MS chromatograms of the mixtures obtained by (A) SFE and (B) methanol extraction, using an ACQUITY UPC² 2-EP Column.

Conventional purification approach: MPLC + HPLC

One of most commonly used approaches in natural product isolation involves MPLC followed by HPLC. In the current case, the SFE extract first underwent a purification step by MPLC (results not shown), attaining the target compound of >97% purity (referred to as the MPLC fraction hereafter). The main remaining impurity has a nominal mass of 360.27 Da, and results from the demethoxylation of the target compound. The structural similarity between the target and impurity presented a challenge in RPLC purification. Figure 2A shows the UPLC-MS and UV chromatograms of the MPLC fraction. A baseline resolution between the target and the impurity was achieved using a 3.0 x 150 mm UPLC column, where the impurity was present as a sodium adduct with an $m/z=383$. The close elution of the two peaks, however, severely hampered the sample loadability in the ensuing RPLC purification. Figure 2B summarizes a loading study of the MPLC fraction on an analytical column (5 μm , 4.6 x 150 mm). The baseline resolution was only preserved with a 10- μL injection. With an 80- μL injection, the impurity peak completely merged into the target peak. In addition to the limited resolution, the elution order

of the compounds also contributed to the low purification productivity. With RPLC, the impurity elutes before the target compound. In the case where target and impurity are partially separated, such as the one with 40- μL injection in Figure 2B, though it is still possible to obtain pure target compound by excluding the front of the target peak where the impurity co-elutes, such practice is generally inadvisable in prep chromatography as the front of a peak often accounts for a high percentage of the total peak. Based on the loading study performed on the analytical column, the maximum loading on a 19 x 150 mm semi-prep column without compromising yield or purity was projected to be 170 μL . At ~ 20 mg/mL, this translates into a maximum loading of 3.4 mg/injection.

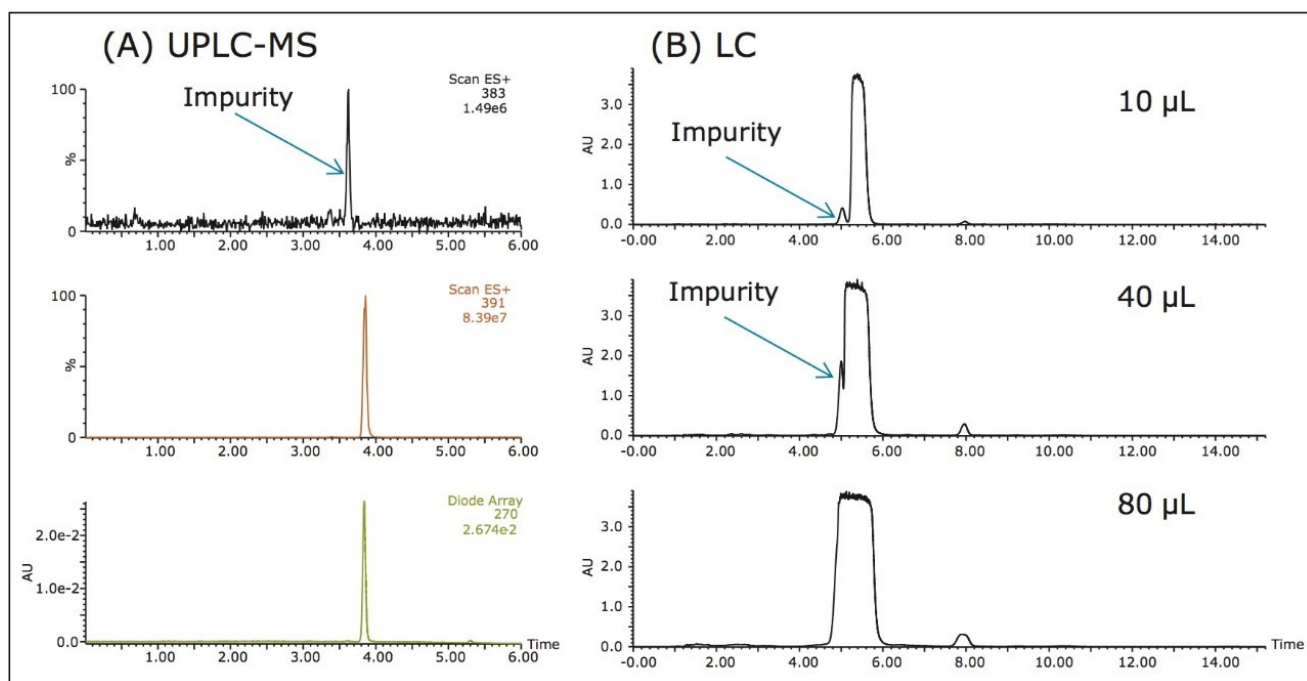


Figure 2. (A) UPLC-MS and UV chromatograms of the MPLC fraction at 1 mg/mL; and (B) LC/UV chromatograms of the MPLC fraction at 20 mg/mL.

Leveraging the orthogonality between RPLC and SFC for improved loading capacity: an MPLC + SFC approach

SFC offers an attractive alternative. SFC is generally considered a normal-phase chromatographic technique when a polar stationary phase, such as 2-EP, is used. As a result, the elution order often reverses that in RPLC using a non-polar C_{18} column. Figure 3A shows the UPC²-MS and UV chromatograms of the MPLC fraction using a BEH 2-EP column. Compared to Figure 2A, not only did the UPC² method provide a better resolution, the elution order of the target and the impurity also reversed. The chromatography was then scaled up to a 19 x 100 mm semi-prep column, and the resulting chromatogram is shown in Figure 3B. The resolution was well maintained with a 600- μL injection at 20 mg/mL. The total run time using SFC was 8 min compared to the 20-

min run time using RPLC. By using prep SFC to replace prep RPLC, the overall productivity was increased by 9-fold; 2.5-fold from the reduced run time and 3.5-fold from the increased sample loading.

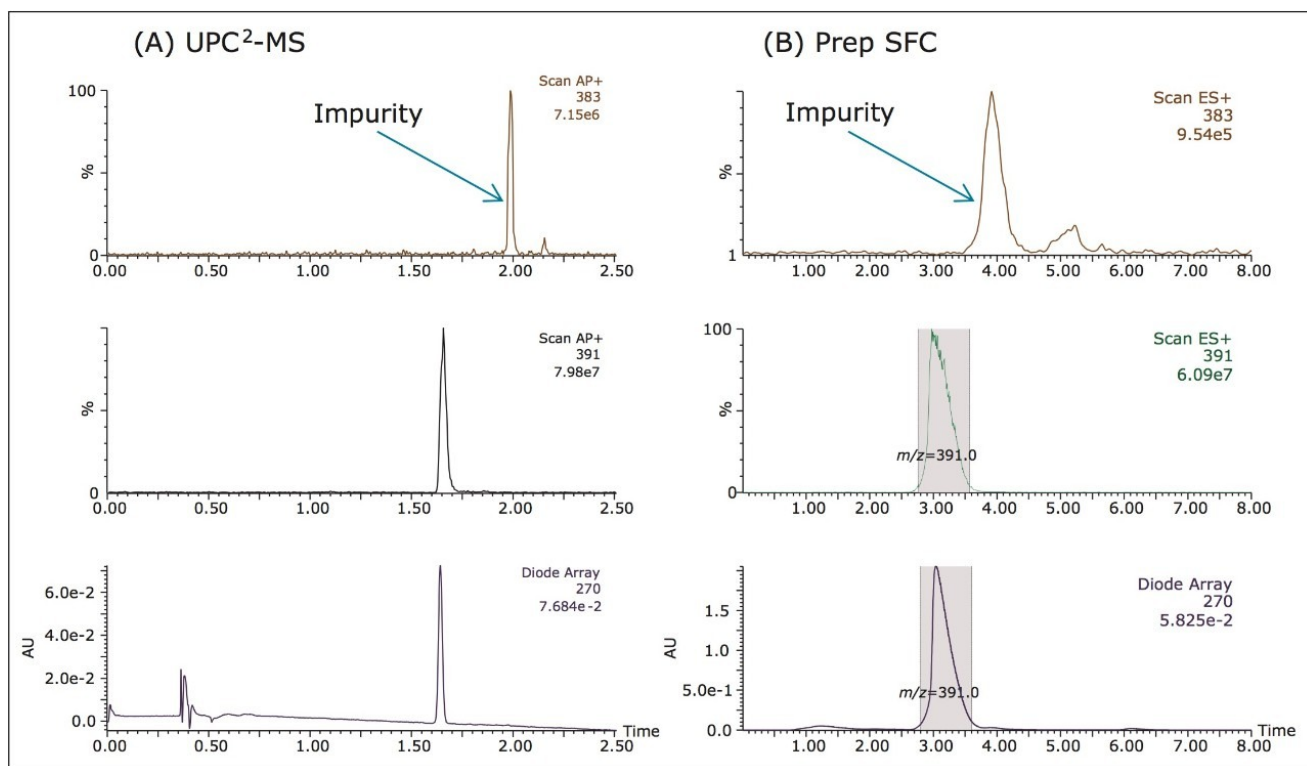


Figure 3. (A) UPC²-MS and UV chromatograms of the MPLC fraction at 1 mg/mL; and (B) prep SFC-MS and UV chromatogram of the MPLC fraction at 20 mg/mL.

Leveraging the orthogonality between different column chemistries in SFC for improved purification productivity: an SFC + SFC approach

Though the approach demonstrated in Figure 3 led to a notable improvement in productivity, the overall process still suffers from large solvent consumption, mainly due to the initial MPLC step. The target compound in the current study has a relatively low polarity. For this sample, a high percentage of organic solvent is required to elute the target compound in LC; hence, the large solvent consumption. In SFC, however, the lipophilic CO₂ is the main mobile phase that elutes the target compound, thus minimizing the use of organic solvents (mobile phase B). Moreover, the raw sample was extracted with neat CO₂ and is, therefore, inherently compatible with SFC.

There is a wide range of column chemistries available in SFC, with retention mechanisms encompassing polar interactions, hydrophobic interactions, π - π interactions, and steric recognitions. With proper selection of column chemistries, SFC can offer orthogonal selectivity necessitated by the sample complexity intrinsic to natural

product isolation. Figure 4 shows the UPC²-MS and UV chromatograms of the SFE extract using a BEH 2-EP (Figure 4A) and a nitro column (Figure 4B), respectively. While 2-EP columns typically render polar interactions between analytes and stationary phase, nitro columns often retain and separate analytes based on π - π interactions. This kind of combination provides complementary separation around the target compound. As can be seen in Figure 4, using a 2-EP column, the target compound at $m/z=391$ is well separated from the impurity at $m/z=361$, but less separated from another later eluting impurity at $m/z=239$. In contrast, using a nitro column, the impurity at $m/z=239$ became an earlier eluting peak and was well separated from the target compound, but the impurity at $m/z=361$ co-eluted with the target compound.

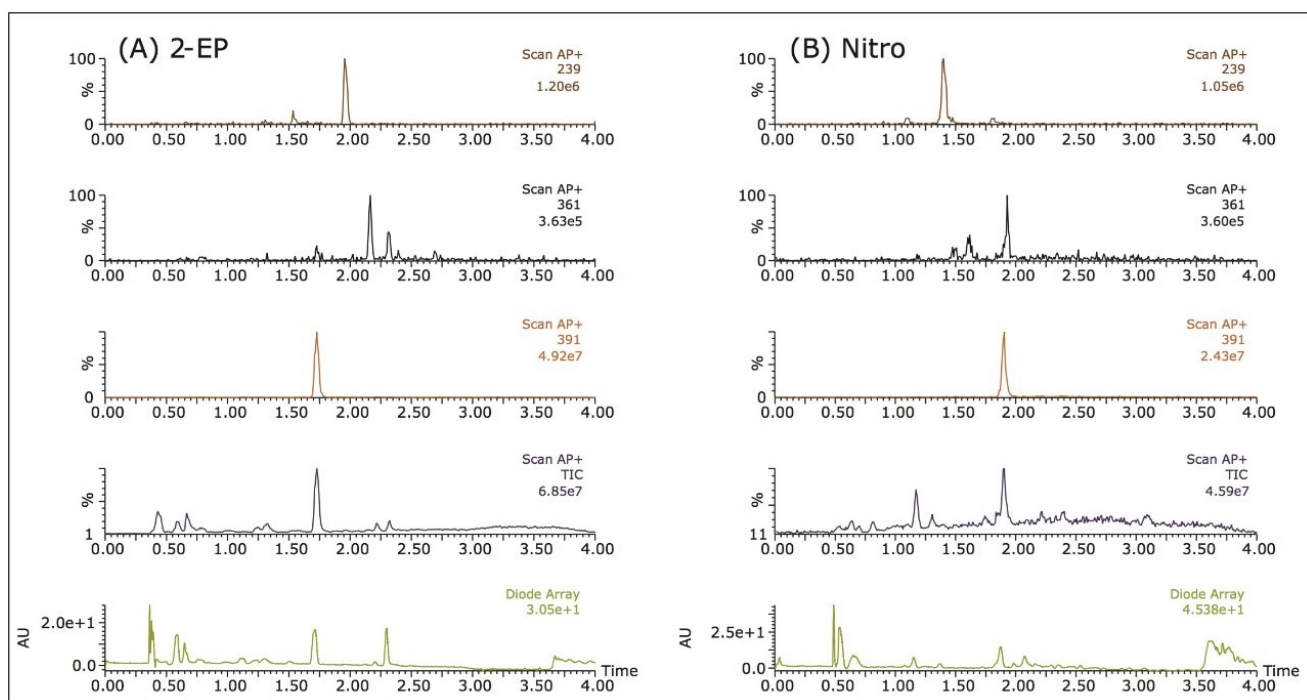


Figure 4. UPC²-MS and UV chromatograms of the SFE extract using (A) a 2-EP; and (B) a nitro column.

Leveraging the orthogonality between different column chemistries in SFC for improved purification productivity: an SFC + SFC approach

Though the approach demonstrated in Figure 3 led to a notable improvement in productivity, the overall process still suffers from large solvent consumption, mainly due to the initial MPLC step. The target compound in the current study has a relatively low polarity. For this sample, a high percentage of organic solvent is required to elute the target compound in LC; hence, the large solvent consumption. In SFC, however, the lipophilic CO₂ is the main mobile phase that elutes the target compound, thus minimizing the use of organic solvents (mobile phase B). Moreover, the raw sample was extracted with neat CO₂ and is, therefore, inherently compatible with SFC.

Based on the retention behavior illustrated in Figure 4, a two-step SFC purification strategy was implemented: using a 2-EP column to remove the main impurity with an $m/z=361$ followed by using a nitro column to remove any remaining impurities after the first step, such as the one with an $m/z=239$. The resulting chromatograms are shown in Figure 5. The overall yield, defined as the weight of the purified pure target compound/the total weight of SFE extract taken for purification, was similar to those from the other two approaches: MPLC+HPLC and MPLC+SFC.

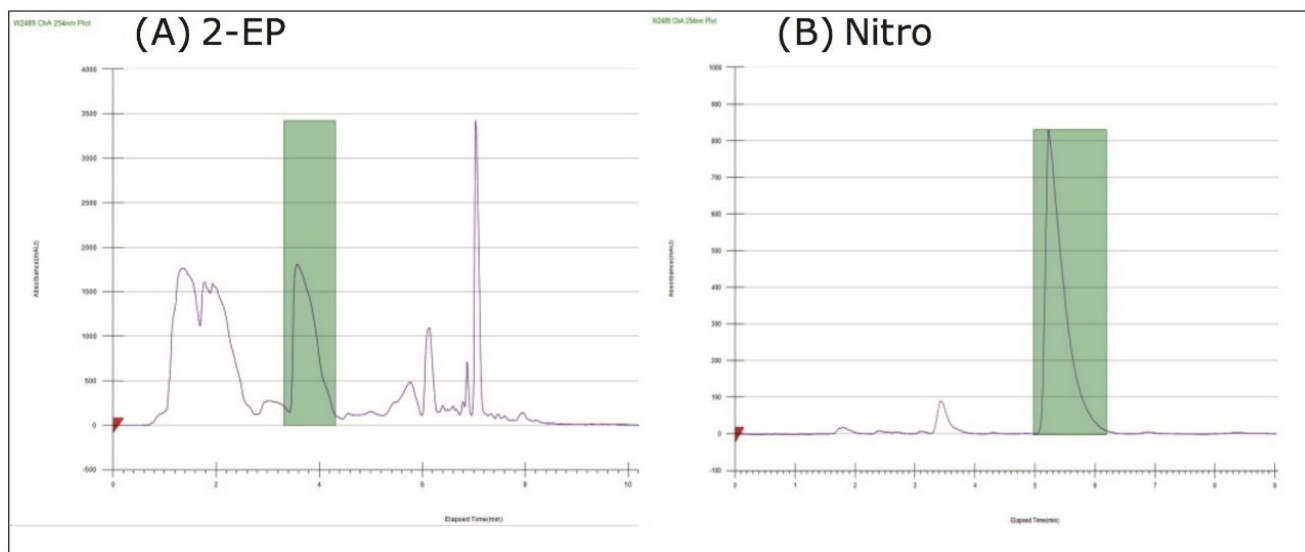


Figure 5. (A) SFC/UV chromatogram of the SFE extract at 133 mg/mL using a Viridis 2-EP column; and (B) SFC/UV chromatogram of the collected fraction from the Viridis 2-EP step on a nitro column.

Aliquots of the purified final product were analyzed by both UPC²-MS and UPLC-MS to ensure a true representation of the sample profile. The resulting chromatograms are shown Figure 6. Both impurities at $m/z=361$ and $m/z=239$ illustrated in Figure 4 were successfully removed. The results indicate that the final product has a purity >99% by UV.

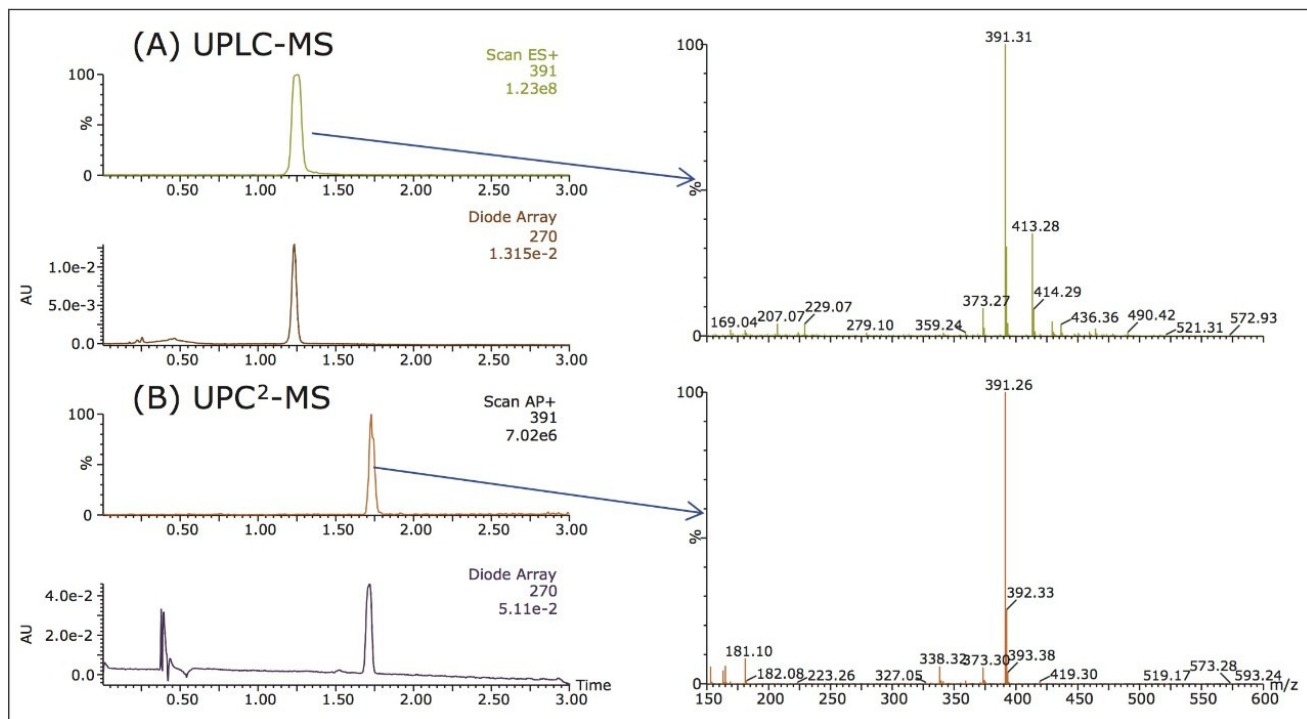


Figure 6. Purity analysis of the final product by (A) UPLC-MS and (B) UPC²-MS.

The SFC purification process resulted in smaller fraction volumes compared to MPLC and HPLC. The SFC fractions were quickly dried under mild conditions, minimizing the possible compound loss due to thermal degradation associated with the post-purification dry-down process. Compared to LC, SFC offered an easier and faster compound recovery.

Process	Rate-limiting step	Productivity (g/24 hr)	Solvent	Solvent consumption (L/24 hr)	CO ₂ use (kg/24hr)
MPLC+ HPLC*	HPLC	0.25	MeOH	95	N/A
MPLC+SFC**	SFC	2.25	MeOH/IPA	75	105
SFC+SFC	First step SFC	3.50	IPA/Ethanol	11	105

*HPLC calculations were based on a 19 x 150 mm column.

**SFC calculations were based on 19 x 150 mm columns.

Table 4. Comparison on productivity and solvent consumption of different purification processes.

Conclusion

In this application note, we have demonstrated employing SFE and prep SFC to holistically improve the productivity in isolating a low-polarity, bioactive compound from a complex natural product

extract. The SFE alleviated the sample complexity prior to analysis and purification, thereby improving sample loading and reducing solvent use in the ensuing chromatography. The SFE extract also lends itself well for SFC analysis and purification.

For the MPLC+HPLC purification route, the target compound and its demethoxylated derivative formed a critical pair in HPLC that limited the column loading and overall purification productivity. The same critical pair was better separated on a 2-EP column using SFC. The elution order of the pair was also altered, enabling an increased column loading. Overall, the MPLC+SFC route offered a 9-fold improvement in productivity. However, both routes still suffered from large solvent consumption because of the MPLC step. Finally, an SFC+SFC purification process was developed, leveraging the orthogonal selectivity between different column chemistries available in SFC. The SFC+SFC route not only led to a 16-fold improvement in productivity, but also a 90% reduction in solvent consumption. In addition, both SFE and SFC also provided an easy sample recovery under mild conditions that minimized potential compound loss due to thermal degradation associated with post-purification dry-down.

The supercritical fluid-based techniques, SFE and SFC, augment the conventional toolbox for natural product research by offering unique selectivity in both extraction and chromatography; and empower laboratories and manufacturers in pharmaceutical, traditional medicine, nutraceutical, dietary supplement, and cosmetic industries for more efficient and more cost-effective natural product isolation and purification.

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