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

John McCauley, Jo-Ann Jablonski, Andrew Aubin, and Rui Chen
Waters Corporation, Milford, MA, USA

APPLICATION 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.

WATERS SOLUTIONS

ACQUITY UPC^2® System

ACQUITY UPLC® H-Class System

AutoPurification™ LC System

Prep 100q SFC MS Directed System

ACQUITY UPC^2 BEH 2-EP Column

Viridis® Silica 2-EP Column

ChromScope™ Software

KEY WORDS

Natural product, purification, prep chromatography, SFE, SFC, UPC^2, cosmetics, selectivity, productivity, orthogonality

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 products.[7]

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 CO_2. The effluent was carried into a 100 mL collection vessel with a makeup flow of 1 min/mL 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 UPC^2-MS experiments were performed on a Waters ACQUITY UPC^2-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.

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 CO_2: 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.

	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 UPC² 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 UPC² 2-EP (1.7 µm, 3.0 x 100 mm)		ACQUITY UPC² 2-EP (1.7 µm, 3.0 x 100 mm)		ACQUITY UPC² 2-EP (1.7 µm, 3.0 x 100 mm)		GreenSep Nitro (1.8 µm, 3.0 x 100 mm)		ACQUITY UPC² 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 UPC.²

	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 CO_2, on the other hand, is a highly lipophilic solvent. As a result, only relatively non-polar compounds are typically extracted by SFE using neat CO_2. 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 UPC^2-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.

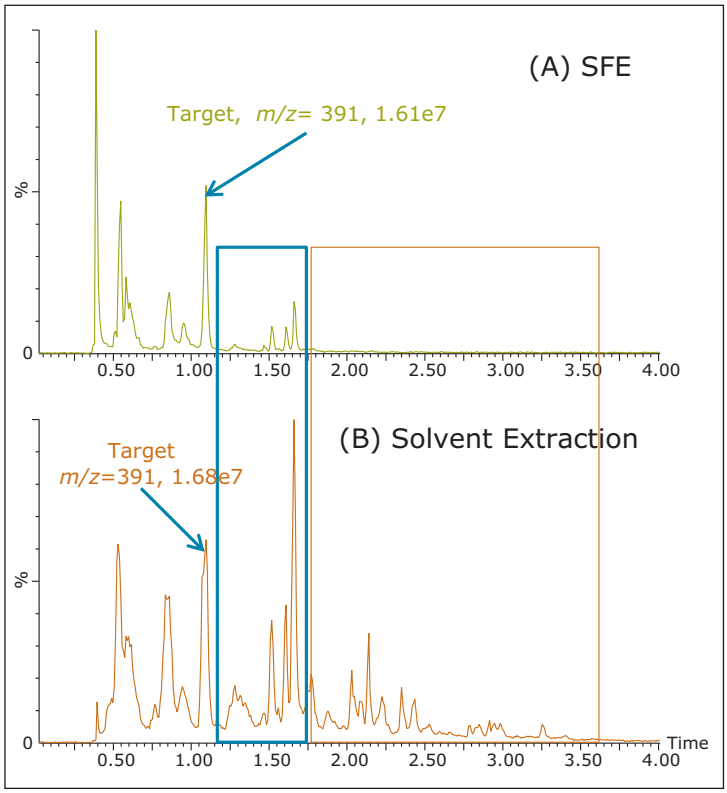

Figure 1. UPC^2-MS chromatograms of the mixtures obtained by (A) SFE and (B) methanol extraction, using an ACQUITY UPC^2 2-EP Column.

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.

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 UPC^2-MS and UV chromatograms of the MPLC fraction using a BEH 2-EP column. Compared to Figure 2A, not only did the UPC^2 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.

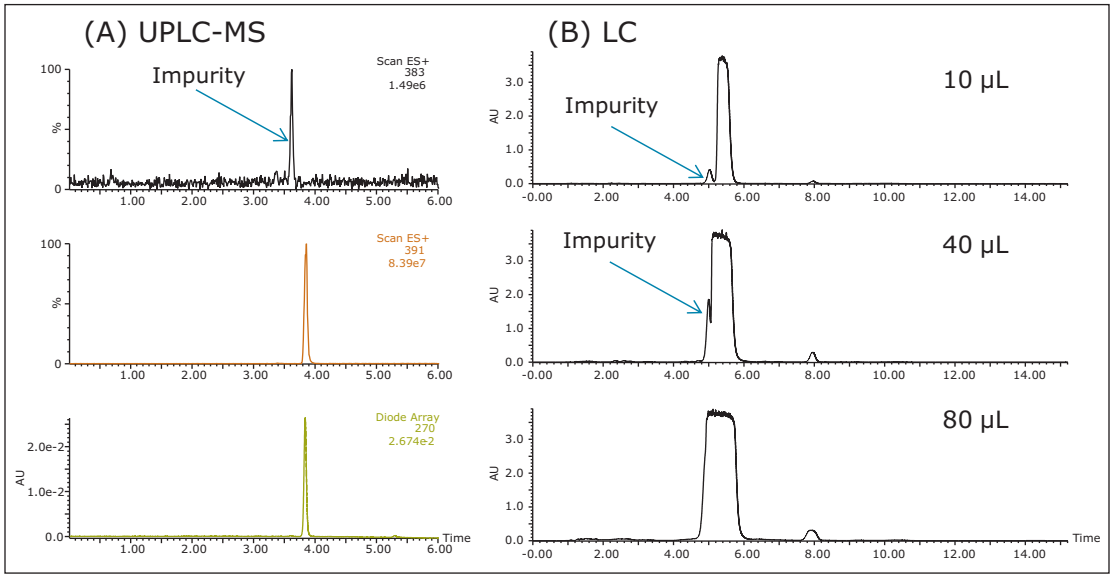

Figure 2. (A) UPLC-MS and UV chromatograms of the MPLC fraction at 1 mg/mL; and (B) LC/UV chromatograms of the MPLC fraction at 20 mg/mL.

Leveraging the orthogonality between 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 CO_2 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 CO_2 and is, therefore, inherently compatible with SFC.

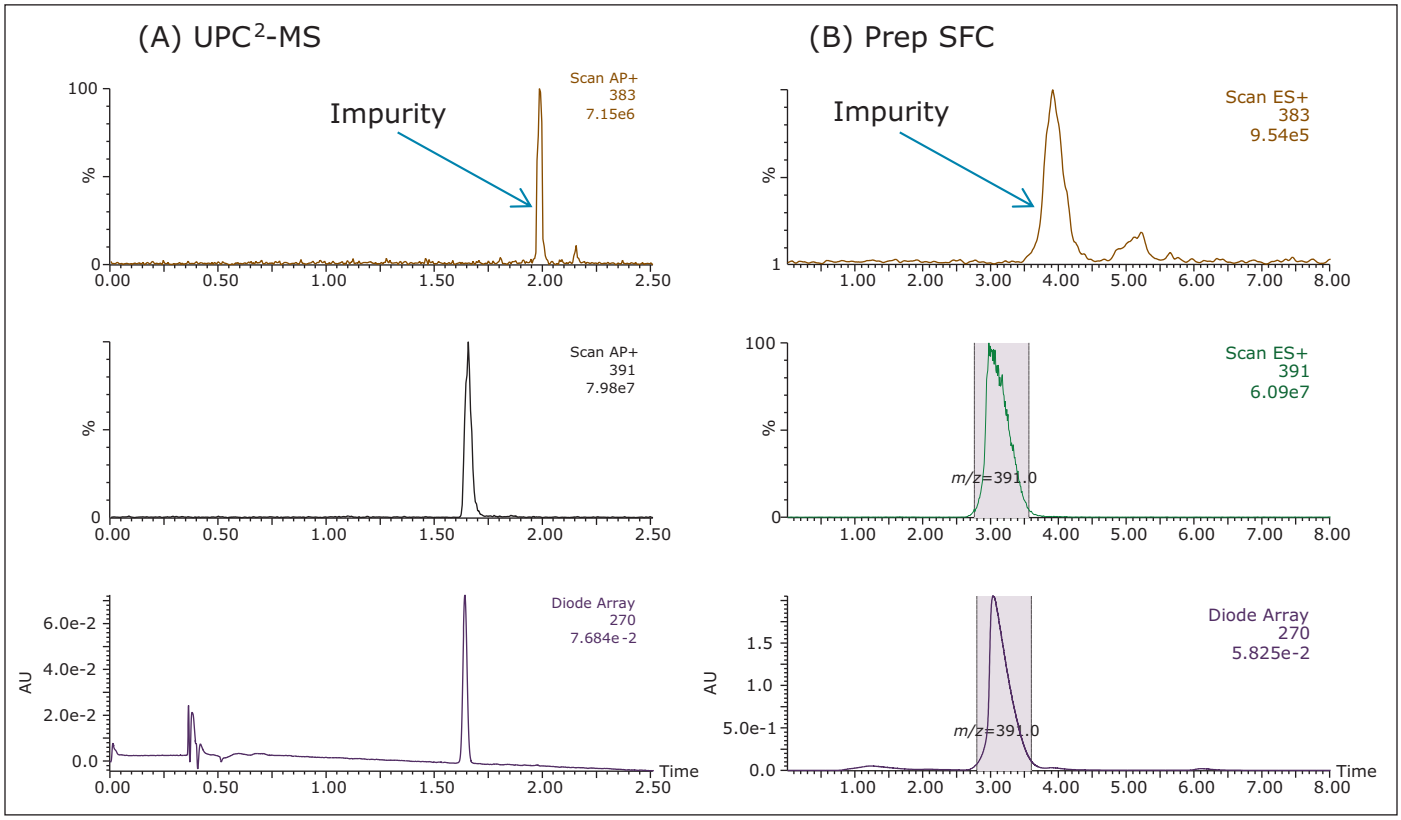

Figure 3. (A) UPC²-MS and UV chromatograms of the MPLC fraction at 1 mg/mL; and (B) prep SFC-MS and UV chromatogram of the MPLC fraction at 20 mg/mL.

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 UPC^2-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.

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

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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.

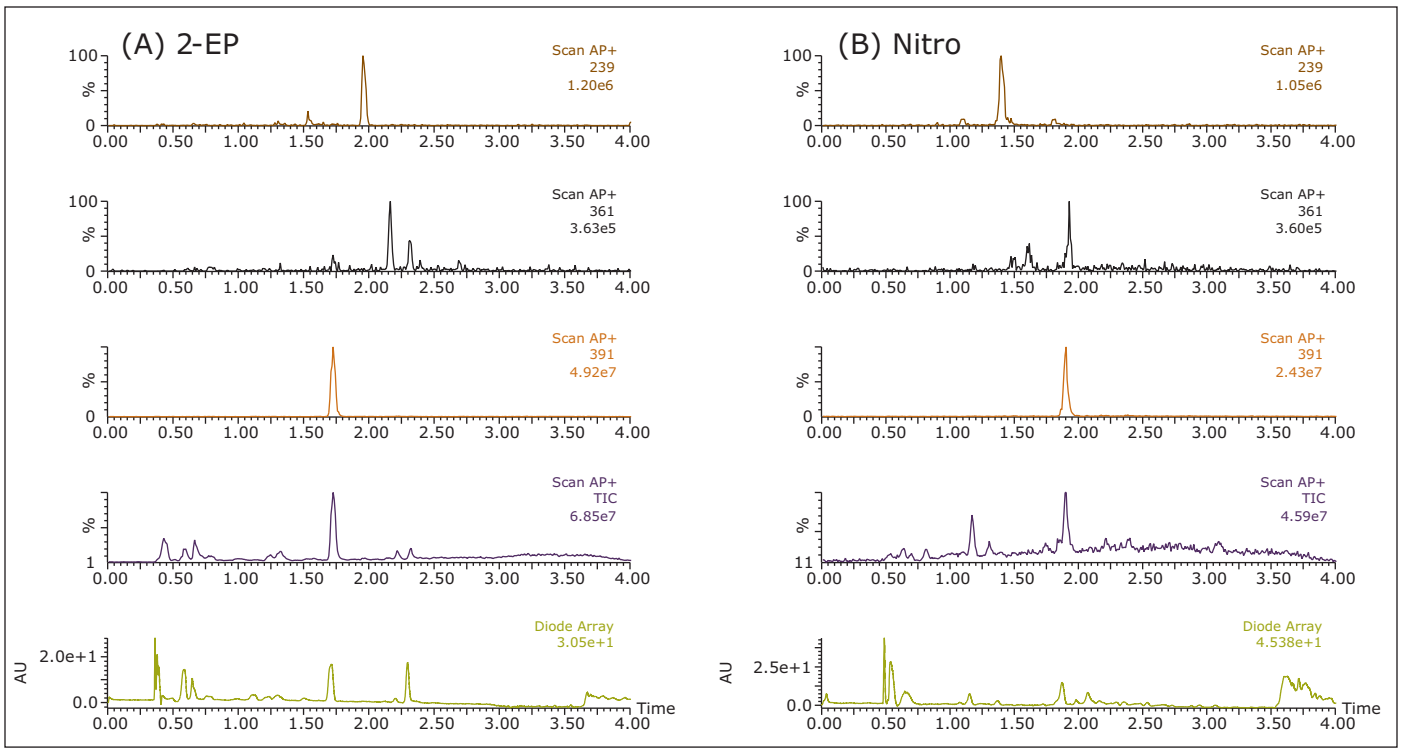

Figure 4. UPC^2-MS and UV chromatograms of the SFE extract using (A) a 2-EP; and (B) a nitro column.

Aliquots of the purified final product were analyzed by both UPC^2-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.

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.

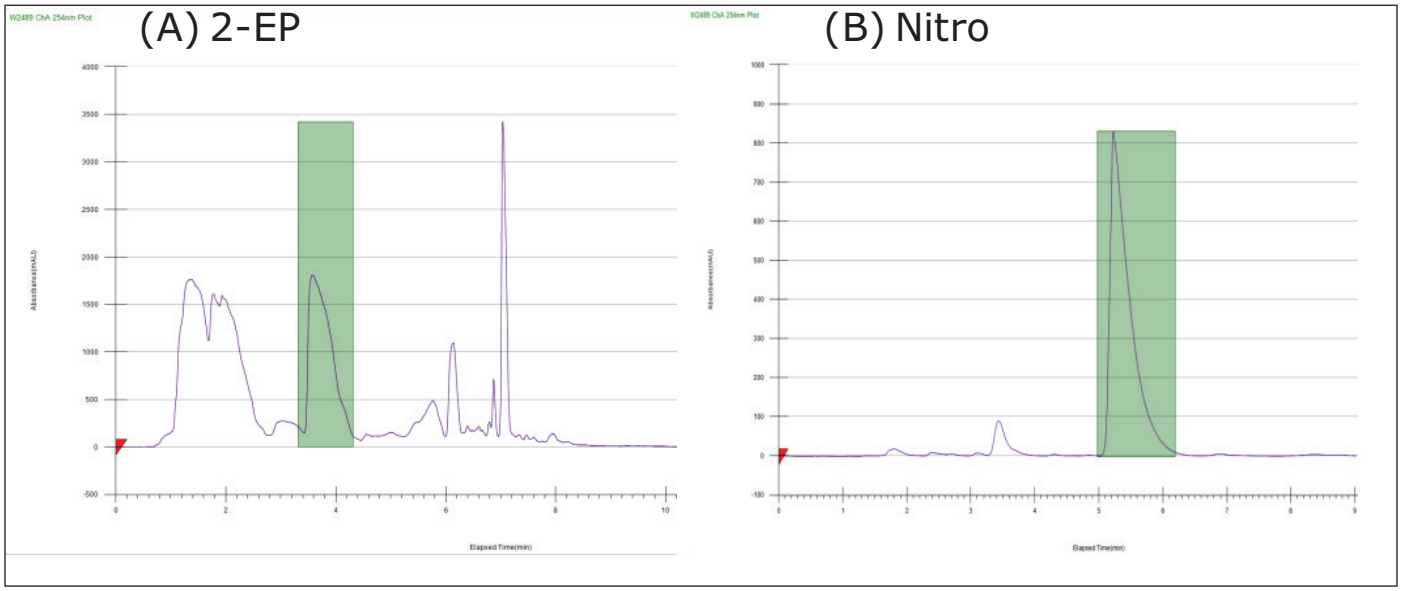

Figure 5. (A) SFC/UV chromatogram of the SFE extract at 133 mg/mL using a Viridis 2-EP column; and (B) SFC/UV chromatogram of the collected fraction from the Viridis 2-EP step on a nitro column.

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.

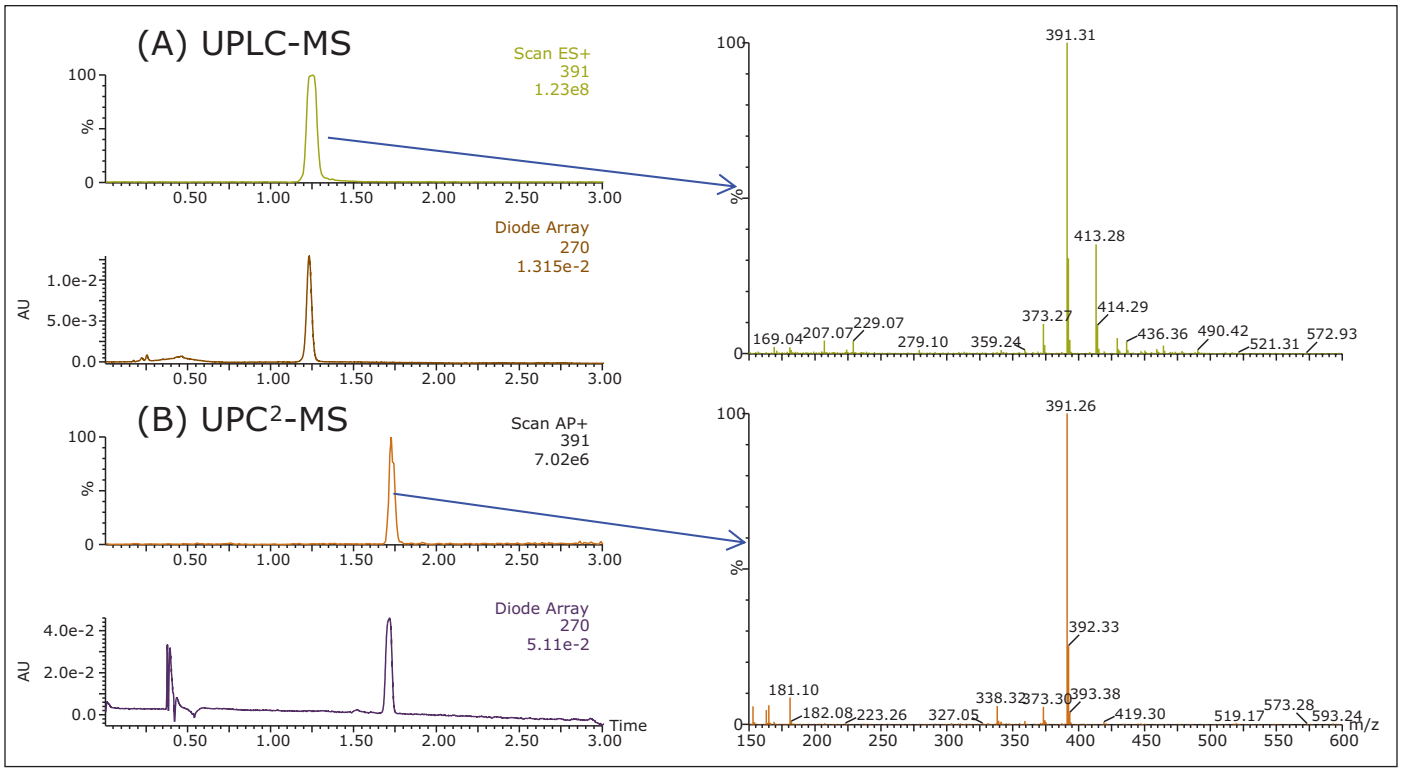

Figure 6. Purity analysis of the final product by (A) UPLC-MS and (B) UPC²-MS.

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.

CONCLUSIONS

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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