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Nota de aplicación

Improving Productivity in Purifying Antroquinonol Using UltraPerformance Convergence Chromatography (UPC^2) and Preparative Supercritical Fluid Chromatography (Prep SFC)

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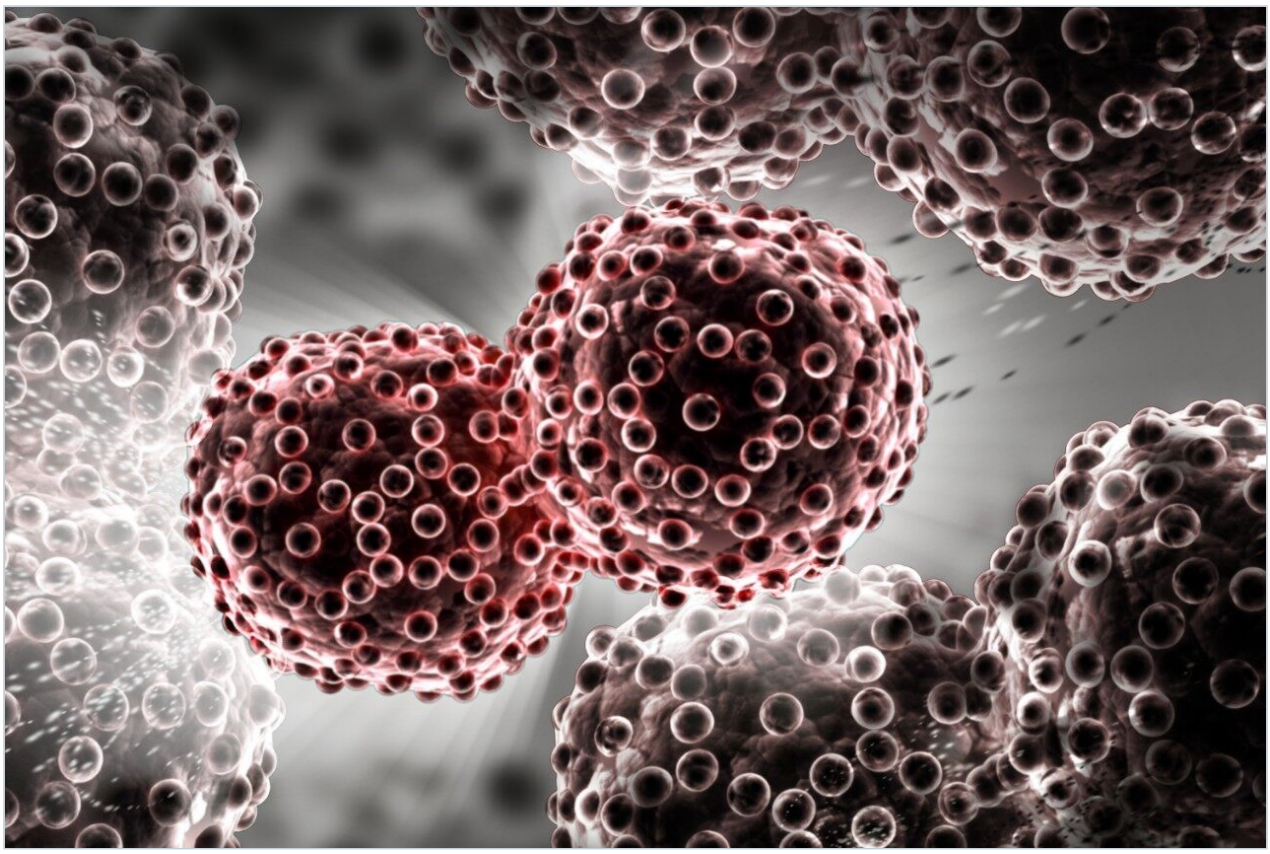

Abstract

Described herein is a comparative study on using prep LC and prep SFC to purify the antroquinonol raw product to achieve >99% purity. Chromatographic behavior of the analytes, including resolution and elution order, using each technique and their implications on downstream preparative chromatography is discussed. The productivity and solvent consumption for each purification technique are also compared.

Benefits

- For antroquinonol and its derivative, UPC^2 results in improved resolution, compared to RPLC, allowing for increased mass loading in the ensuing prep SFC method.
- UPC^2 and prep SFC methods yielded a more favorable elution order compared to RPLC, further facilitating the purification step due to increased mass loading.
- Purification via prep SFC offered a nine-fold improvement in overall productivity and reduced the organic solvent use by 77% compared to the prep HPLC approach.

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 used directly in the forms of food supplements, nutraceuticals, and alternative medicines.[5] Isolation and purification of bioactive compounds play an important role in natural product research. The most commonly used process involves extraction of target compounds from the cellular matrix and pre-purification by various low to medium pressure liquid chromatographic techniques, predominantly reversed-phase liquid chromatography (RPLC).[6] While being a generally applicable chromatographic technique for a variety of compound classes, RPLC does not guarantee adequate resolutions for all analytes, especially for those 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.[7] To that end, supercritical fluid based chromatographic techniques, including UltraPerformance Convergence Chromatography (UPC^2), a novel analytical chromatographic technique that applies the performance advantages of UPLC to supercritical fluid

chromatography (SFC), and preparative supercritical fluid chromatography (prep SFC) have brought viable new additions to the natural product research toolbox by offering a wide range of selectivity complementary to RPLC.

Antroquinonol, with its structure shown in Figure 1, is a ubiquinone derivative recently isolated from the mycelium of Antrodia camphorata, a parasitic fungus unique to Taiwan.[8] Antroquinonol has proven cytotoxic activities against multiple tumor cell lines.[9-11] Pre-purification of antroquinonol from the mycelium extract involves two RPLC steps of using silica gel and size exclusion gel, respectively, resulting in a raw product of 98% purity.[9] In order to support medicinal research where a higher purity (>99%) product is generally required, it is imperative to develop an efficient and cost-effective purification strategy to further purify the raw product. Described herein is a comparative study on using prep LC and prep SFC to purify the antroquinonol raw product to achieve >99% purity. Chromatographic behavior of the analytes, including resolution and elution order, using each technique and their implications on downstream preparative chromatography is discussed. The productivity and solvent consumption for each purification technique are also compared.

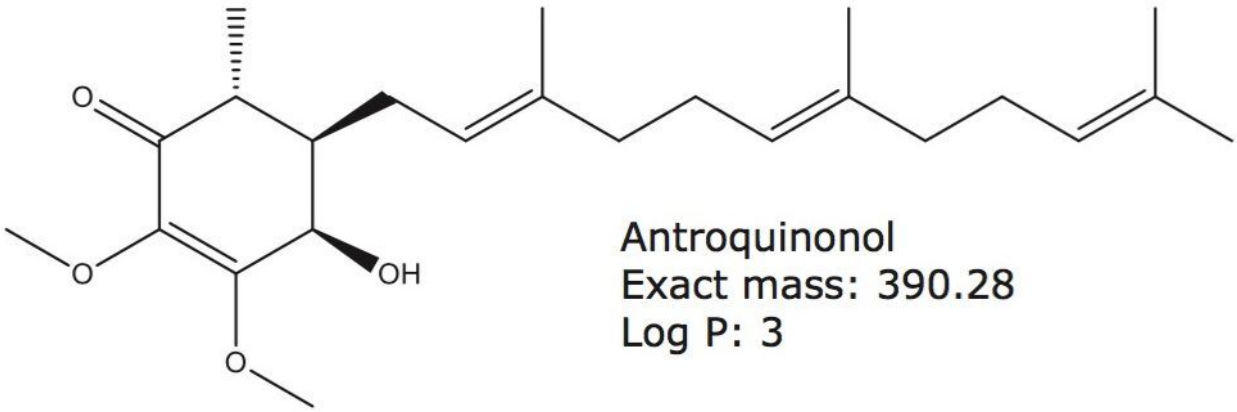

Figure 1. The chemical structure, molecular mass, and Log P of antroquinonol.

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-Aldrich (St. Louis, MO, USA). The

antroquinonol raw product (98% purity) was from a commercial source and dissolved in methanol at 1 mg/mL for analytical experiments and 20 mg/mL for preparative experiments.

Chromatography

The UPLC-MS experiments were performed on a Waters ACQUITY UPLC H-Class with SQ Detector 2. The UPC^2-MS experiments were performed on a Waters ACQUITY UPC^2 with ACQUITY TQD Mass Spectrometer. Both systems were controlled by MassLynx Software. The LC-MS experiments were performed on a Waters AutoPurification LC System with 3100 Mass Detector. The preparative SFC experiments were performed on a Waters Prep 100q SFC System with 3100 Mass Detector. Both systems were controlled by MassLynx Software and FractionLynx Application Manager. Detailed experimental parameters are summarized in Tables 1 and 2.

	Figure 2A		Figure 2B		Figure 4A	
Instrument	ACQUITY UPLC H-Class/ SQD 2 System		AutoPurification LC MS System		ACQUITY UPLC H-Class/ SQD 2 System	
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 UPLC HSS T3 (3.0 x 150 mm, 1.8 µm)		Atlantis T3 (4.6 x 150 mm, 5 µm)		ACQUITY UPLC BEH C_{18} (2.1 x 50 mm, 1.7 µm)	
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 LC.

	Figure 3A		Figure 3B	
Instrument	ACQUITY UPC^2 /ACQUITY TQD		Prep 100q SFC System with 3100 Mass Detector	
Flow rate (mL/min)	1.5		80	
Mobile phase A	CO_2		CO_2	
Mobile phase B	Isopropanol		Isopropanol	
Backpressure (psi)	1740		1740	
MS detection	APCI+		ESI+	
Temperature (°C)	45		40	
Injection volume (µL)	1		600	
Column	ACQUITY UPC^2 2-EP (3.0 x 100 mm, 1.7 µm)		Viridis Silica 2-EP (19 x 150 mm, 5 µm)	
Gradient	Time (min)	%B	Time (min)	%B
	0	5	0	5
	2.50	25	1	5
	2.75	40	6.5	9
	3.25	40	7	9
	3.50	5	7.25	5
	4	5	8	5

Table 2. Key experimental parameters for UPC^2 and Prep SFC.

Results and Discussion

Figure 2A shows the UPLC-MS chromatogram of the antroquinonol raw product. The peak at *m/z* 391 is the sodium adduct of antroquinonol and the impurity peak at *m/z* 383 is the sodium adduct of the demethoxylated antroquinonol. Although baseline resolved, the structural similarity between antroquinonol and its derivative resulted in a rather limited resolution, which severely hampered the sample loadability in the prep LC. Figure 2B summarizes a loading study of the raw product on an analytical column (4.6 x 150 mm, 5 µm). The baseline resolution was only preserved with a 10-µL injection. The resolution deteriorated as the injection volume increased, and completely diminished with an 80-µL injection. If geometrically scaled up to a 19 x 150 mm semi-prep column, the maximum loading is projected to be 170 µL. At 20 mg/mL, this translates into a maximum loading of 3.4 mg/injection.

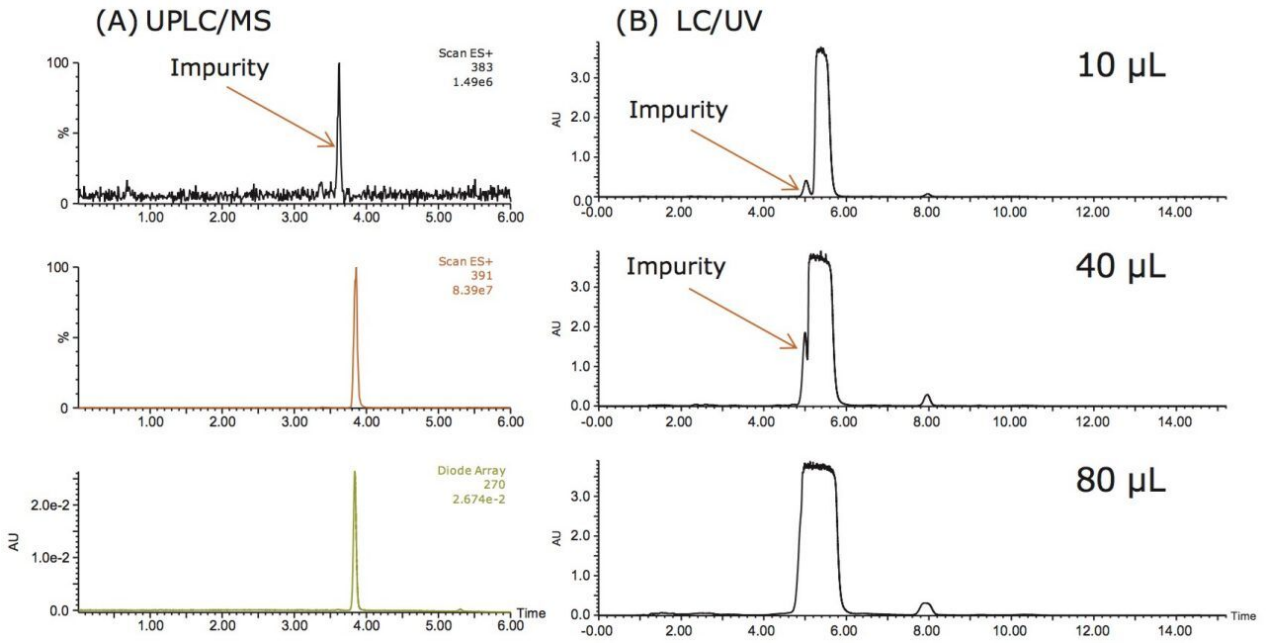

Figure 2. (A) UPLC-MS of the raw antroquinonol product at 1 mg/mL and (B) LC/UV chromatograms of the raw antroquinonol product at 20 mg/mL.

UPC^2 offers an attractive alternative. Figure 3A shows the UPC^2-MS chromatogram of the antroquinonol raw product. Compared to UPLC (Figure 2A), the UPC^2 method provided a better resolution between antroquinonol and its derivative, allowing for an increased mass loading in the ensuing prep chromatography. It is also noted that the elution order of antroquinonol and its derivative is the opposite of that in RPLC. When a polar stationary phase, such as 2-EP, is used, UPC^2 resembles normal phase chromatography (NPLC) and offers orthogonal selectivity to RPLC. As a result, the elution order of the analytes is often the reverse of that in RPLC. Elution order could play an important role in the overall productivity of prep chromatography, especially for those closely eluting target/impurity pairs. Since the peak front generally accounts for a higher weight percentage of the total peak than the peak tail of the same time interval, it is highly desirable to have the target compound elute before the impurity, so that when the target and impurity are less than baseline resolved, only a small portion of the target peak is excluded during collection. In the current study using RPLC, the impurity elutes before the target. With a 40-µL injection (Figure 2B), high purity antroquinonol can only be obtained at the expense of target recovery and total productivity. In contrast, the prep SFC chromatograms depict a much more favorable scenario for prep chromatography (Figure 3B). With impurity eluting after the target, high purity antroquinonol can be collected with negligible loss in productivity, even at the loading level where antroquinonol and the impurity slightly overlap, as shown in Figure 3B.

The UPC^2 method was scaled up to a 19 x 100 mm semi-prep column. Based on the chromatographic behavior shown in Figure 3A, a focused gradient ranging from 5 to 9 B% was used. The resulting chromatogram is shown in Figure 3B. The total run time was 8 min, compared to the 20-min run time using RPLC. The maximum loading was empirically determined to be 600 µL. At 20 mg/mL, this represents a 12 mg/injection.

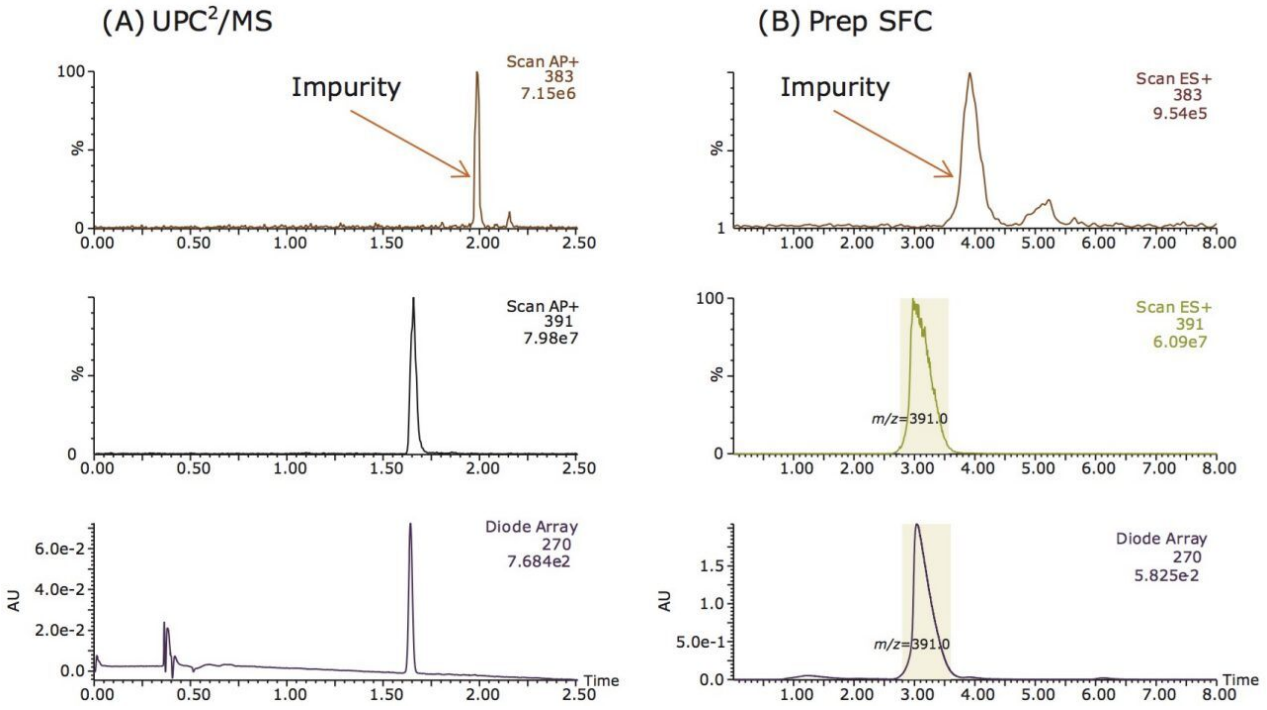

Figure 3. (A) UPC^2-MS of the raw antroquinonol product at 1 mg/mL and (B) prep SFC-MS chromatogram of the raw antroquinonol product at 20 mg/mL.

Aliquots of the purified antroquinonol product were analyzed by UPC^2/PDA-MS and the results are shown in Figure 4. The main impurity at *m/z* 361 was successfully removed, as shown in the corresponding mass spectrum. The final antroquinonol product has a >99% purity by UV at 270 nm.

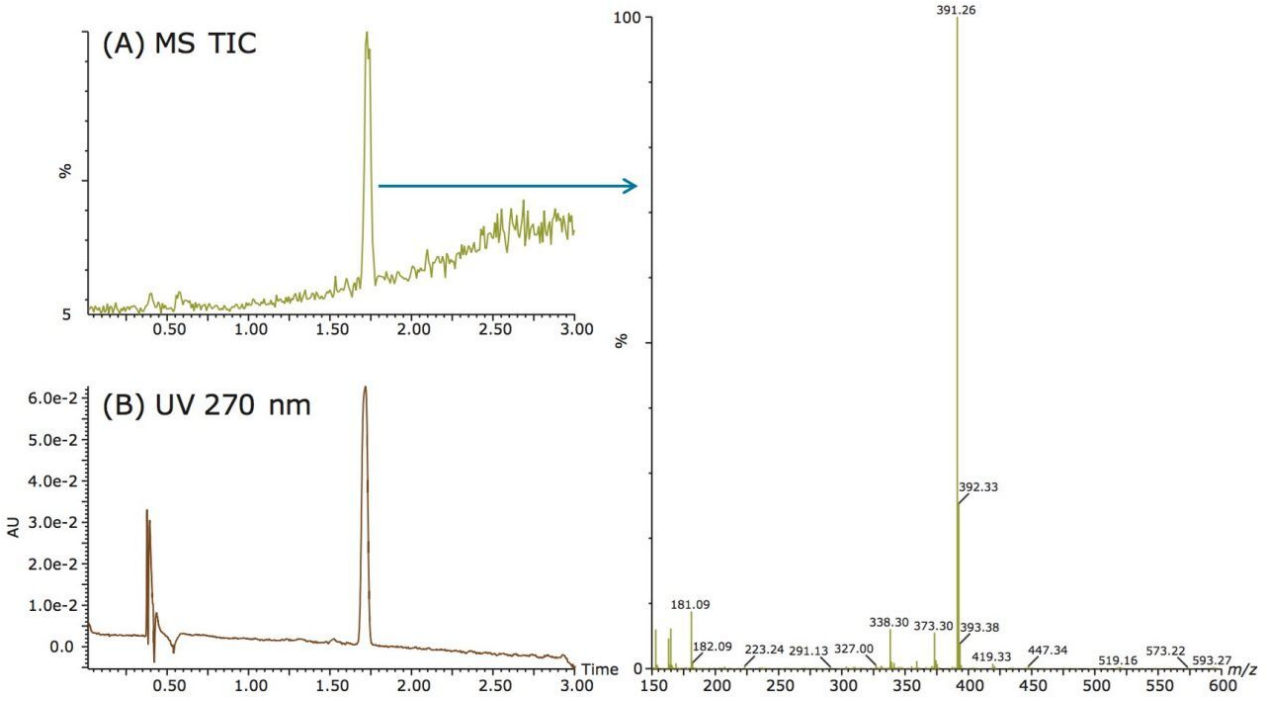

Figure 4. Purity analysis of the final antroquinonol product by UPC^2 with UV and MS detection.

A comparison on the productivity and solvent consumption was summarized in Table 3. Overall, by using prep SFC to replace prep RPLC, the purification productivity was increased by nine-fold with the following breakdown: 2.5-fold from the reduced run time and 3.5-fold from the increased sample loading. The organic solvent use was also reduced by 77%.

Prep chromatographic technique	Productivity (g/24 hr)	Solvent	Organic solvent consumption (L/24 hr)	CO_2 use (kg/24 hr)
HPLC	0.25	MeOH	33.52	N/A
SFC	2.25	MeOH/IPA	7.70	105

Table 3. Comparison on productivity and solvent consumption of two purification approaches.

Conclusion

Two different chromatographic approaches to purify a raw antroquinonol product to the desired 99% purity

have been demonstrated. In the HPLC approach, the critical pair of antroquinonol and its demethoxylated derivative resulted in a limited resolution; hence, limited mass loading in prep chromatography and limited purification productivity. The same critical pair was better separated, and had a more favorable elution order versus RPLC, using Waters UPC^2 and Prep 100q SFC technologies, allowing for an increased mass loading in prep SFC when the analytical UPC^2 method was scaled up. Overall, the prep SFC approach offered a nine-fold improvement in productivity and reduced the organic solvent use by 77% compared to the prep HPLC approach. The supercritical fluid-based techniques, UPC^2 and prep SFC, augment the conventional toolbox for natural product research by offering complementary selectivity to RPLC, and enable laboratories and manufacturers in pharmaceutical, traditional medicine, nutraceutical, and dietary supplement industries with more efficient and more cost-effective natural product purification.

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