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Supercritical fluid chromatography eliminates chlorinated solvents to purify natural products

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Supercritical fluid chromatography eliminates chlorinated solvents to purify natural products

Abstract

Although supercritical fluid chromatography (SFC) has been used for achiral purification, including for natural products, the potential for SFC to replace chlorinated solvents for chromatography has been overlooked. SFC is becoming more commonly used, but its use is hindered by its reputation for being difficult to run that chemists are unfamiliar with the technique. Because many chemists are unfamiliar with SFC, the *Introduction* reviews SFC and compares it to liquid chromatography. Modern SFC instruments are as easy to use as flash chromatography systems, with the various controls for creating stable supercritical conditions being handled entirely in software, even during gradients. Modern SFC instruments are very stable, allowing reproducible results like high performance liquid chromatography (HPLC) systems. There are many substitutes for chlorinated solvents for liquid chromatography, which are evaluated with thin layer chromatography (TLC), but SFC needs only a few pure co-solvents to produce acceptable purifications. Although silica chromatography often requires solvent modifiers for good results, SFC allows other column packings than silica, as well as co-solvent additives. Scouting gradients allows facile method development. Several examples of natural products purified using column chromatography with chlorinated solvents are shown. Some examples suggest that natural products purification may be done without chlorinated solvents.

Keywords: SFC, pharmacognosy, method development, chlorinated solvents, HPLC

Introduction

There are many reasons to consider SFC in place of chlorinated solvents for chromatography. Although generally used for chiral purifications, SFC works very well for achiral purification, including the use of stacked injections to purify large amounts of compound. For one, the carbon dioxide used for SFC is recycled from other processes, allowing it to be reused instead of simply vented to the atmosphere. Another

is ease of use. SFC has a reputation for being difficult to use. However, newer systems have computer-controlled back-pressure regulators and flow meters that make them as easy to use as flash chromatography systems and as reproducible as HPLC systems. Method development is now very easily done too. Evaporation time is reduced because carbon dioxide is removed during fractionation.

Chlorinated solvents, particularly dichloromethane, are commonly used for non-SFC[1] chromatography because they can dissolve a wide variety of polar and non-polar compounds. The UV cutoff of these solvents allows detection at 254 nm. These solvents are also easily evaporated.

Problems with chlorinated solvents

Chlorinated solvents have long been known to be toxic; hazards include carcinogenic effects, organ toxicity, reproductive damage, and neurotoxicity[1]. For this reason, chlorinated solvents are being phased out in the United States[2,3] and elsewhere[4].

To reduce the use of chlorinated solvents, particularly dichloromethane (DCM), TLC was used to evaluate other solvents, such as heptane and methyl or ethyl acetate or heptane and acetone, to replace them[5]. Another reference[6] suggested replacing DCM with *tert*-butyl acetate, *sec*-butyl acetate, ethyl isobutyrate, and methyl pivalate as the weak solvents. The strong solvent was either methanol or a mixture of 3:1 ethyl acetate: methanol. MacMillan *et al* [7] used methanol paired with cyclopentyl methyl ether, 2-methyl tetrahydrofuran, dimethyl carbonate, and *tert*-butyl methyl ether along with ethyl acetate. Another comparison of relative eluting strengths of DCM replacements containing solvent modifiers[8] also used TLC to evaluate elution. Thin layer chromatography was used to evaluate various solvent combinations and compositions because there is a direct correspondence between retention on a TLC plate and on a column with a matching stationary phase[9].

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4 Although there have been many solvents tested for normal phase
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6 chromatography, SFC as a technique has not heretofore been evaluated as a replacement
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8 for chlorinated solvents. Although SFC has been used to purify natural products, there
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10 has not been a study showing that SFC can replace the use of chlorinated solvents. Li *et*
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12 *al* [10] listed a summary of compound classes derived from plants purified with SFC,
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14 along with a description of columns and co-solvents. Noticeably absent for the list of
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16 co-solvents are chlorinated solvents. Hartmann [11] wrote a review describing
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18 purification of natural products with SFC and listed only a single example using
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20 chlorinated solvents. Another review by Gibitz[12] showed the different classes of
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22 natural products purified by SFC, and no examples listed therein used chlorinated
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24 solvents. The experimental section will show examples of compounds purified with
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26 chlorinated solvents with liquid chromatography but purified without those solvents
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28 with SFC. Although there are relatively few instances of silica columns being used in
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30 the cited reviews, this stationary phase is very useful for SFC[13], so its use is explored
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32 here.
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35 36 37 ***Differences between SFC and liquid chromatography*** 38

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40 Although SFC is a type of normal phase chromatography, its roots are derived from gas
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42 chromatography[14]. As the origins of SFC are different, some of the nomenclature is
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44 different from liquid chromatography. Nearly all modern SFC instruments use carbon
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46 dioxide as the weak solvent. The strong solvent which moves the sample down the
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48 column is called a “modifier” or “co-solvent.” The term “co-solvent” is used herein to
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50 reduce confusion with the modifiers used for pH control or ion-pairing in liquid
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52 chromatography. An “additive” in SFC plays the role of “modifier” as used in liquid
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54 chromatography. An SFC instrument converts carbon dioxide to a supercritical state
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56 which is pumped through a column. After the purification, the carbon dioxide is
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removed with a gas-liquid separator, and the compounds are collected in the co-solvent with a fraction collector.

Despite these differences, SFC uses columns that are also used for liquid chromatography. The software for controlling the instrument is very similar to that used for liquid chromatography instruments. It has settings for temperature and back pressure that control the density of the supercritical solvent, but users generally use the factory default values.

Disadvantages of SFC

Unlike liquid chromatography, SFC cannot use TLC directly for method development. Carbon dioxide is supercritical, or subcritical, under pressure (55 Bar) and moderate temperature, which precludes the use of TLC chambers.

SFC method development

SFC is normal phase, as gradients run from less polar (carbon dioxide) to more polar conditions. The columns are generally more polar than the solvents used. Compared with liquid chromatography, SFC allows the pressure, and thus the density, of the mobile phase to be changed. Higher back pressures increase the density of the mobile phase, which generally causes the sample to be more soluble in the mobile phase, decreasing retention time. The back pressure need not be changed for most purposes. SFC systems usually employ a column heater as well, which works the same as those in liquid chromatography systems. The temperature affects the mobile phase density, with higher temperatures reducing the density of the supercritical mobile phase; this tends to increase retention time. The vast majority of purifications work with the default settings on the preparative SFC, which are used for the analytical unit as well. Simply using the default settings makes the SFC as easy to use as a liquid chromatography system.

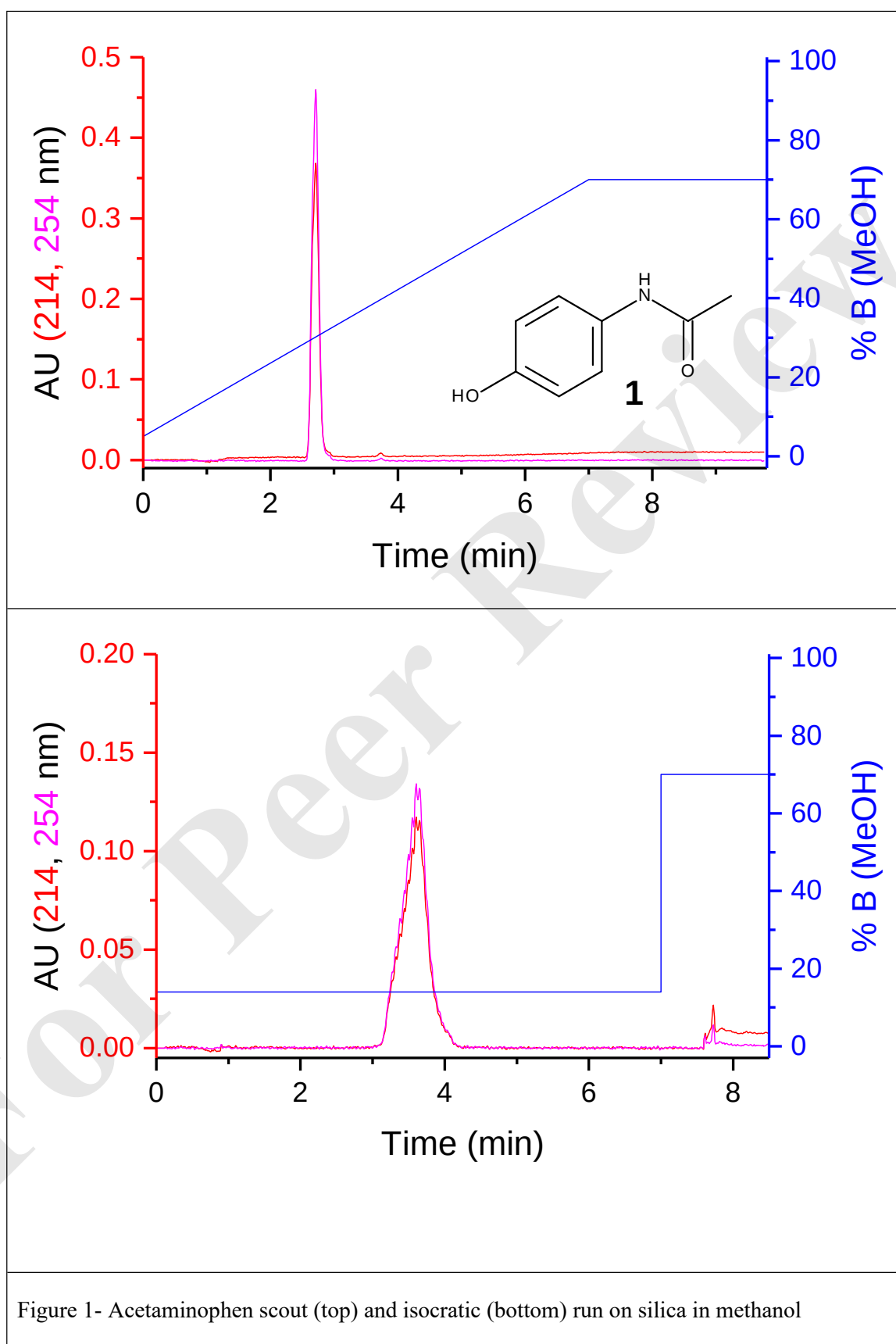
TLC method development and SFC

TLC allows screening of several different solvent systems in a short time, but several plates are needed to screen solvents. TLC plates do not exist for several SFC column packings. The applicability of TLC to SFC method development ranges from simple correlation of the co-solvent to retention on SFC[15] to very little correlation[16]. There is little predictive power for TLC when applied to SFC.

Scouting gradients and SFC method development

However, it is possible to perform method development on the SFC system using scouting gradients on either preparative or analytical SFC systems. The scouting gradients allow different columns and solvents to be screened for resolution or to determine whether the compound will elute. Scouting runs take the same amount of time as a TLC plate.

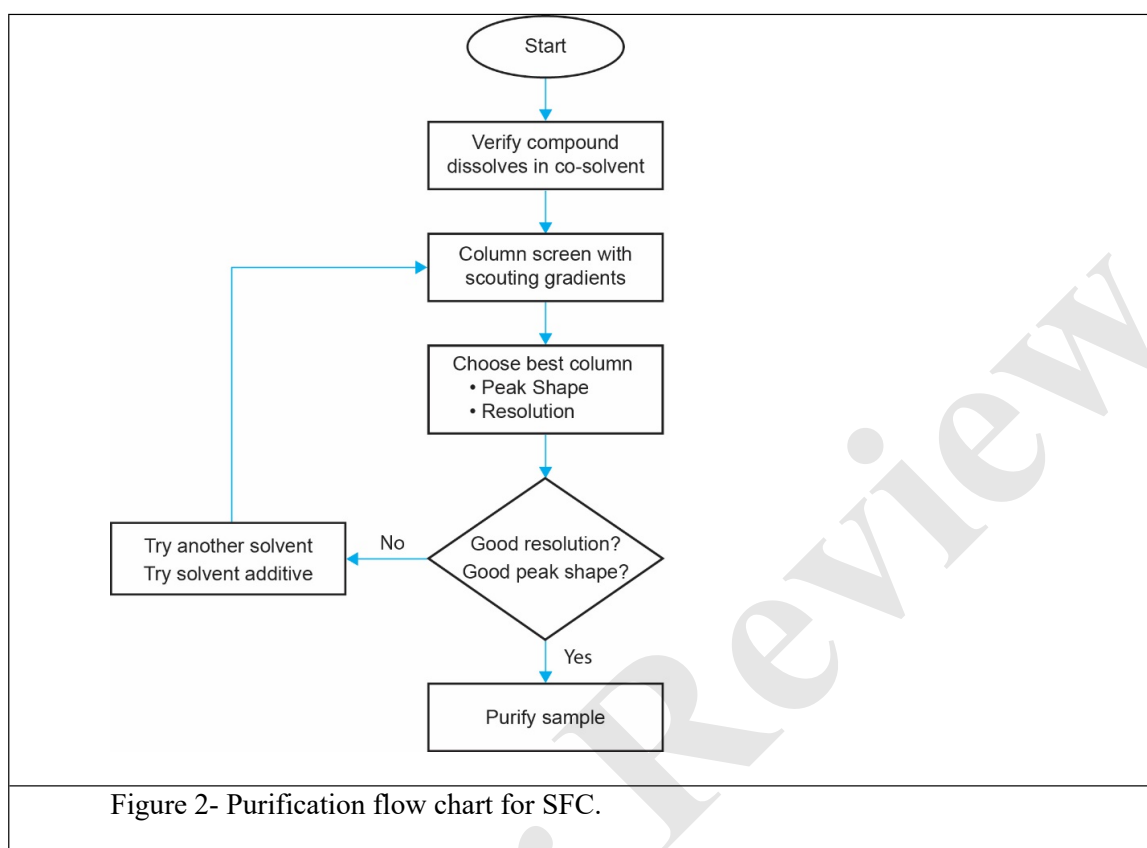
Scouting gradients are used to determine elution methods for liquid chromatography[17] by correlating retention in a calibrated scouting gradient to create nearly isocratic methods. Such gradients work the same for SFC as for liquid chromatography, as demonstrated in *Figure 1*, where acetaminophen was run on a silica column using methanol. Acetaminophen is one compound used to evaluate replacements for chlorinated solvents for chromatography[18].



Scouting runs also provide additional information beyond calculating efficient purification methods. The detectors can often provide a spectrum that suggests the best wavelength for peak collection to maximize recovery. Mass spectrometers allow verification of the correct compound for synthesized samples. Scouting gradients have also been used in “Assay Directed Method Development” in concert with biological assays to create efficient purification methods for natural products[19]. This technique uses scouting gradients in which fractions are collected during the entire gradient; these fractions are evaluated for biological activity. The retention time for the active fractions is then used to determine the focused gradient for purification. In natural products, the bioactive compound(s) are often unknown and so, as in TLC, a scouting solvent system is chosen to spread the peaks out to allow resolution from inactive peaks. A preparative column is useful to collect fractions for assay screening during the scouting run.

For good results, the sample should be soluble in the SFC co-solvent; otherwise, sample loading will be limited, causing small peaks, or broad peaks.





Just as TLC plates may need to be run with several solvent combinations to determine a good composition to resolve the compounds, scouting gradients can be run with different solvents and stationary phases to get a good resolution per Figure 2. A good starting point is silica using methanol as the co-solvent. To improve resolution, a weaker solvent might be chosen, such as ethanol, isopropanol, or even ethyl acetate, provided that the sample is soluble in those solvents. Peaks that show fronting or tailing may need a solvent modifier such as diethyl amine (DEA). However, changing the column to a different type such as diamine may also help. The results section will show examples of method development.

Materials and Methods

All preparative SFC runs used an ACCQPrep SFC and RediSep Prep columns (20x150

mm) (both from Teledyne ISCO, Lincoln NE, USA). Scouting runs used either the ACCQPrep SFC or an Agilent 1260 analytical SFC system (Agilent Technologies, Santa Clara, CA, USA) equipped with RediSep Prep analytical columns (4.6x150 mm). The analytical scouting gradients were run from 5 to 50% B for 10 minutes at a flow rate of 3.00 mL/min. The column temperature was 40 °C, and the back pressure regulator (BPR) was set to 100 Bar. Scouting runs on the ACCQPrep SFC employed the 20x150 mm columns that were then used for the preparative focused or isocratic gradient. Scouting gradients were run from 5 to 70% co-solvent. The flow rate was 60 mL/min, and the column oven temperature was set to 40 °C. Silica columns were used for several runs prior to the runs described herein, as the retention and selectivity of compounds often shift during the first few runs with alcohols. The retention stabilizes within a few runs. Such a retention change is due to either water adsorption into the alcohol and then onto the column[20] or the formation of silyl ethers[21]. All preparative methods were determined from the scouting runs, from either the analytical SFC or the preparative system, using the Focus Gradient feature in PeakTrak software in the ACCQPrep SFC, which is based on the Time-on-Target algorithm[17]. All the compounds and extracts listed in the experimental and result sections were listed as purified using chlorinated solvents in literature references. Pure compounds were purchased from Midland Scientific (La Vista, NE USA). Reverse phase runs used RediSep Gold C18 flash columns with a CombiFlash NextGen 300 (Teledyne LABS).

Results

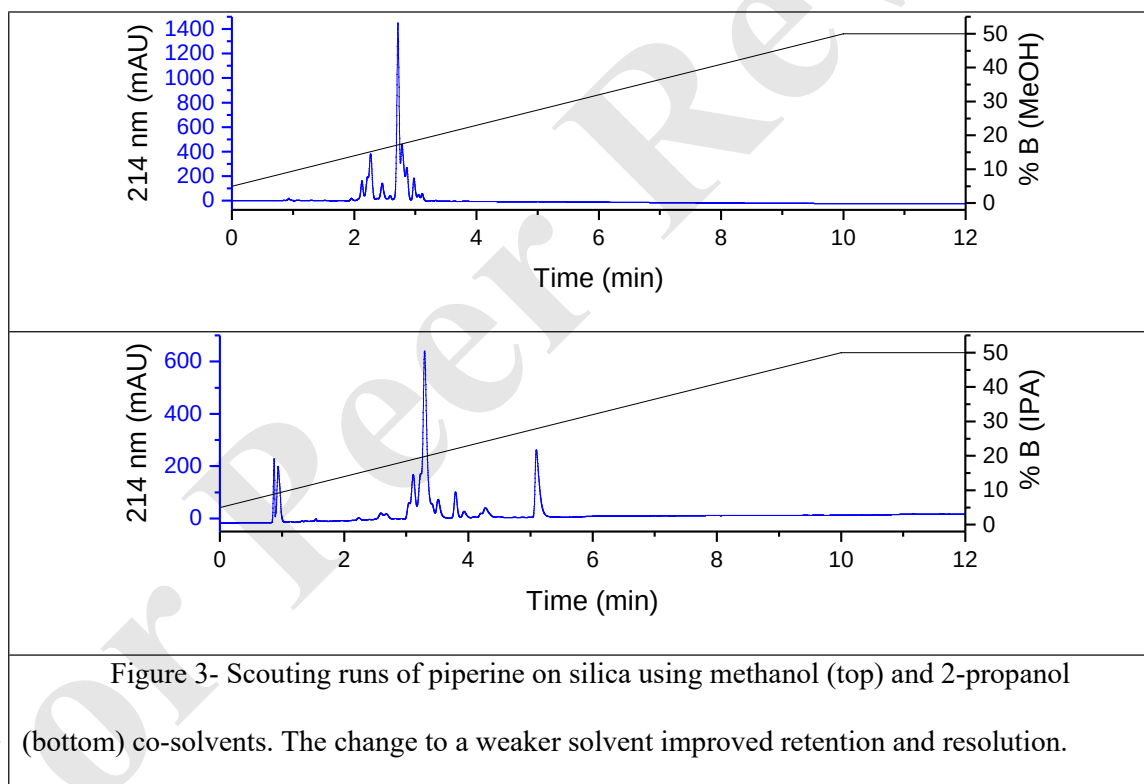
Examples of extract purification workflows

Piperine

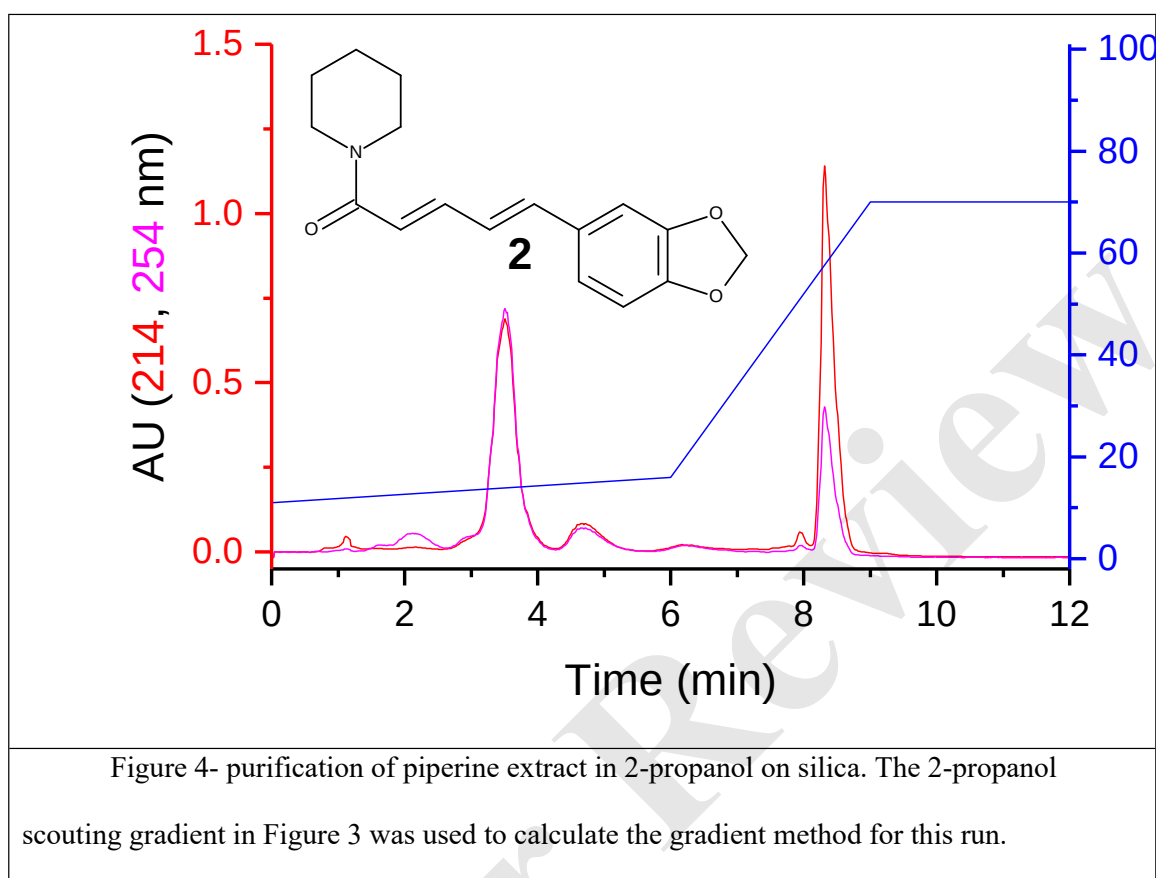
Piperine is a compound that was purified with a chloroform/methanol gradient using

silica gel[22]. As it is extracted with supercritical carbon dioxide, it is no surprise it can run in an SFC system. For this experiment, black pepper balls (20 g, Swad, Raja Foods, 8110N. St. Louis Ave, Skokie IL 60076) were ground to a powder and extracted with dichloromethane. The extract was washed with water containing 1 N hydrochloric acid. The aqueous washings were adjusted to pH 10 with ammonium hydroxide and extracted with dichloromethane to yield a 19 mg sample that was used without further purification.

Scouting runs used the analytical SFC for method development. Methanol was used for the initial trial, and 2-propanol was used for the second one (Figure 3).



The 2-propanol co-solvent showed improved resolution and was used for the preparative purification (Figure 4). The loading for this run was 18.5 mg.



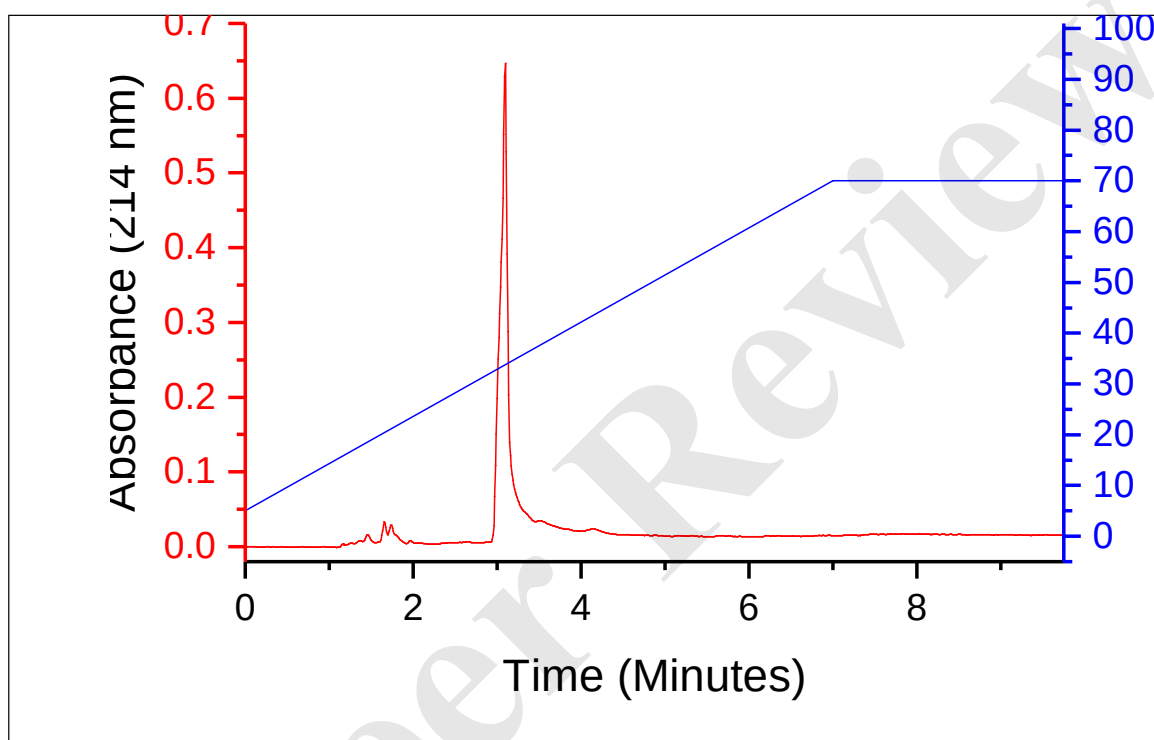
A reference of pure piperine was used to verify the elution of piperine from the column at 3.5 minutes. The scouting run allowed easy determination of a gradient method.

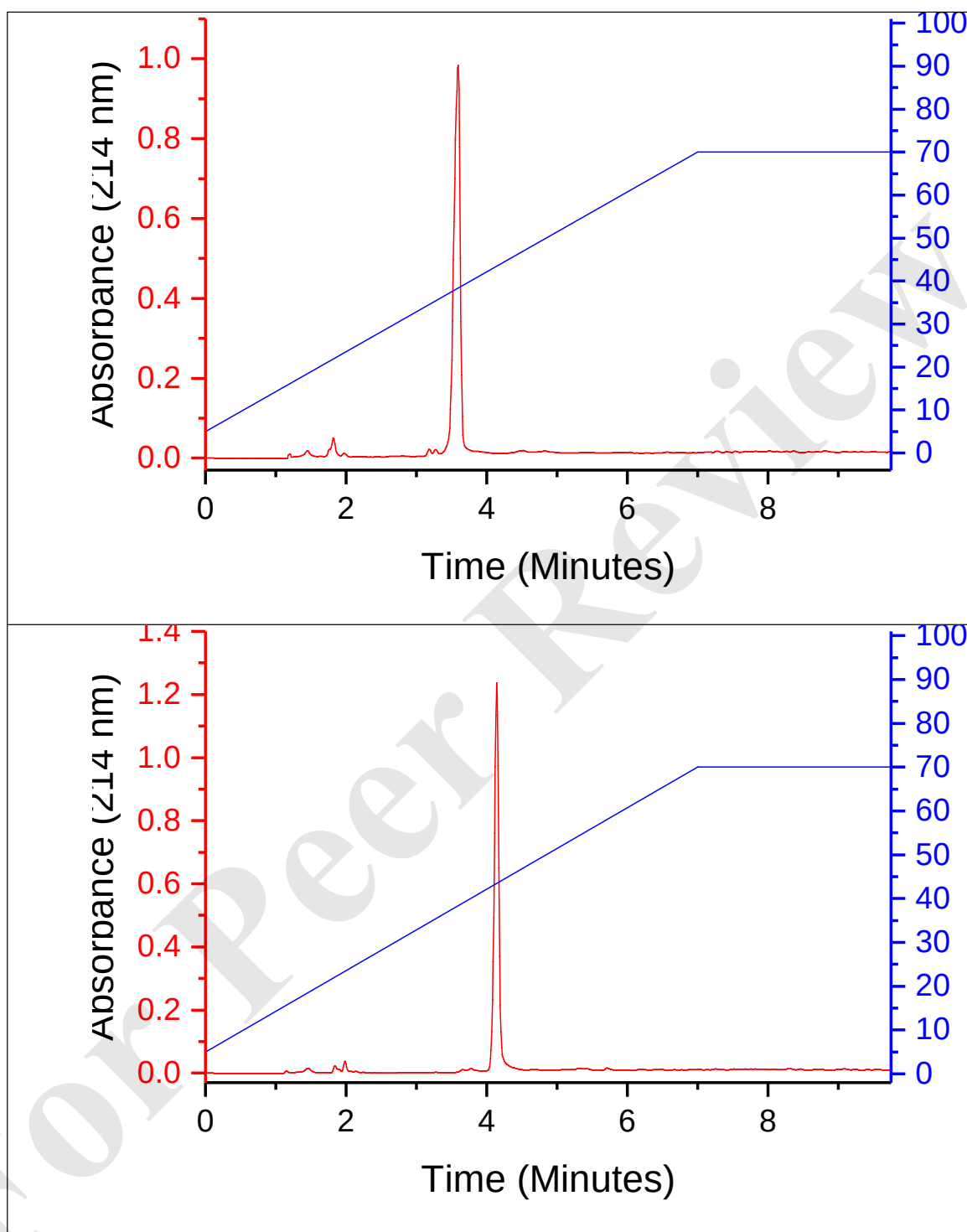
Curcumin compounds

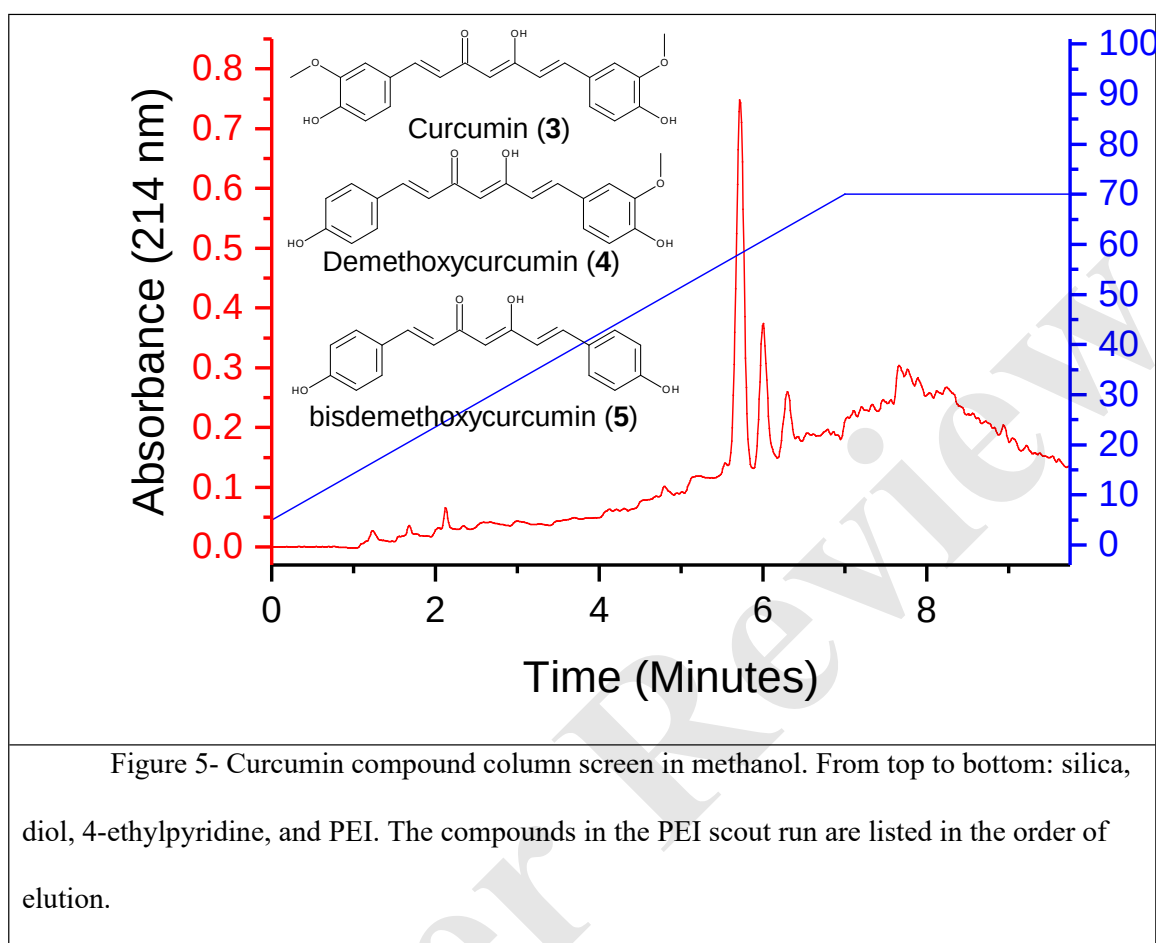
Curcumin contains several components that are also purified by chloroform/methanol silica chromatography[23]. Curcumin was extracted and purified by flash chromatography (CombiFlash NextGen 300 with RediSep C18AQ flash column, water/methanol 0.1% formic acid).

After flash purification, scouting gradients were used with a column screen to find the best purification conditions. In this case, the scouting gradients were run on the preparative SFC system. One reason is that this system can reliably run up to 70% co-

solvent. All columns except the PEI column showed the mixture as a single peak using methanol with no additive. Curcuminoids have already been purified with SFC using a BEH column, but they needed an oxalic acid additive[24] in methanol as compared with the PEI column, which used neat methanol.







The Focus Gradient Generator in the ACCQPrep SFC was used to calculate an isocratic method, which was then used to create stacked injections to purify the compounds.

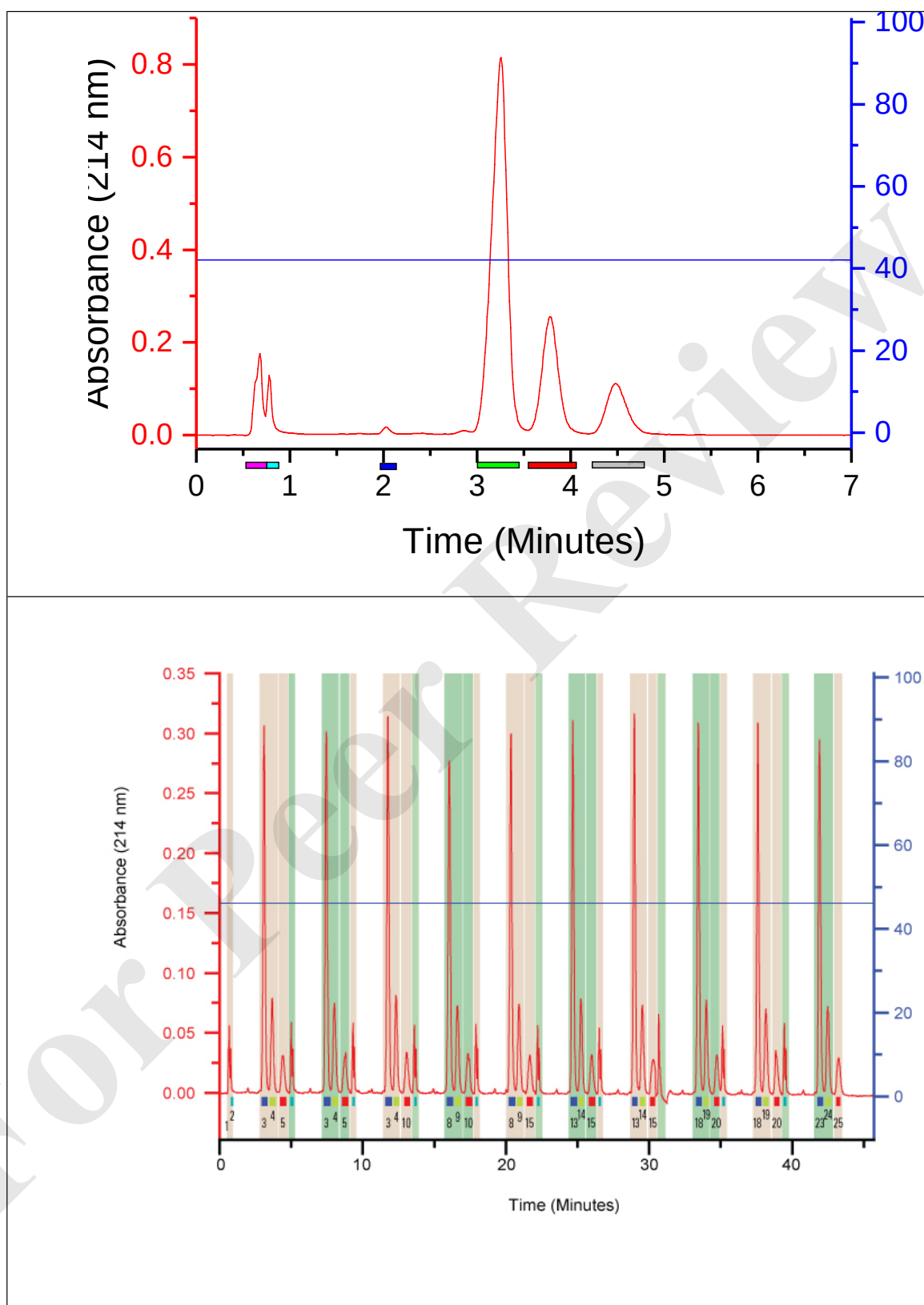
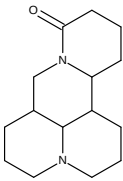


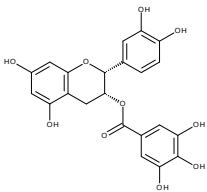
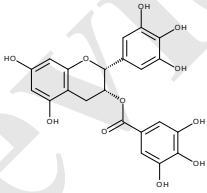
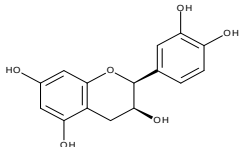
Figure 6- Isocratic method (top) and stacked injection used to rapidly purify a mixture of cumin compounds.

Evaluation of other compounds purified with chlorinated solvents

The examples in this section are natural products that were listed in the literature as being purified using chlorinated solvents in liquid chromatography. Pure compounds were purchased and run on SFC to determine if they could be run on SFC without chlorinated solvents, and under what conditions they would run.

Compound[citation]	Column Focus gradient range	solvent	Chlorinated solvent used in original purification	Structure
matrine[25]	Silica Broad peak in scout run	methanol, no additive	Dichloromethane with methanol	 6
	Diol Broad peak in scout run	methanol, no additive		
	4-ethylpyridine 18.1-28.1	methanol, no additive		
	PEI 26.6-36.6	methanol, no additive		
	Silica 30.7-40.7	methanol 0.5% isopropyl amine		

oxymatine[25]	Silica No elution	methanol	Dichloromethane with methanol	7
	Diol Late elution, severe tailing	methanol		
	4-ethylpyridine 34.3- 44.3	methanol		
	PEI Some tailing	methanol		
	Silica 58.0-68.0	methanol 0.5% isopropyl amine		
cytisine[25]	Silica- very broad peak	methanol	Dichloromethane with methanol	8
	Diol Broad peak	methanol		
	4-ethyl pyridine 17.5 – 27.5	methanol		
	PEI 26.7-36.7	methanol		
	Silica 28.4-38.4	methanol 0.5% isopropyl amine		

(-)-Epicatechin 3-gallate[26]	Silica 24.7 -34.7	methanol	Dichloromethane with methanol	 9
	Diol Peak tailed badly	methanol		
(-)-Epigallocatechin 3-gallate[26]	Silica Wide, tailing peak in scout	methanol	Dichloromethane with methanol	 10
	Diol 38.4-48.4	methanol		
	Silica 25.8-38.8	Methanol 0.5% FA		
Epicatechin	Silica 19.0- 29.0	methanol	This is commonly isolated with other catechins and was run to demonstrate it could be resolved from them.	 11
	Silica 18.2-28.2	Methanol 0.5% FA		
Table 1- compounds purified via column chromatography using chlorinated solvents and run on SFC. Compounds showing acceptable peak shape for a column and co-solvent have a focused gradient range listed.				

All the compounds run here were purified with methanol in conjunction with chlorinated solvents for liquid chromatography but were run with simple alcohols either on a column which showed good peak shape or on silica with an additive. Those

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4 compounds that are acidic ran well on silica with a formic acid additive, whereas basic
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6 compounds ran well with an isopropyl amine (IPAm) additive. IPAm was used because
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8 it is relatively easily removed by evaporation. Other additives include methanol
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10 containing ammonia, dimethyl amine, and triethyl amine (TEA). As these additives may
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12 interfere with mass spectrometers, using one of the other columns is an option for SFC-
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14 MS. Although the bioactive compound is commonly unknown until it is purified, the
15
16 isolation steps provide clues to its nature. Alkaloids are commonly purified by partition
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18 with acidic water, followed by partition into an organic solvent by making the aqueous
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20 solution basic, so these are candidates for purification using silica with a co-solvent
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22 with a basic additive, or using PEI, diamine, or ethyl pyridine columns with a cosolvent
23
24 with no additives.
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27 Most of the compounds listed in this paper were purified with methanol
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29 combined with chlorinated solvents; however, hexanes (or another hydrocarbon) is
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31 commonly used in conjunction with another chlorinated solvent. Acetaminophen
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33 (compound 1), depicted in *Figure 1*, eluted with only DCM on TLC[18], showing that
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35 less polar compounds can be run. If a compound elutes too early using methanol, a
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37 weaker co-solvent can be used such as 2-propanol (as demonstrated in *Figure 3* with
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39 piperine (2)) or ethyl acetate. Up to 5% water can be used with methanol for compounds
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41 that bind strongly to silica, or silica can be replaced with diol. This is exactly analogous
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43 to normal phase liquid chromatography. Ethanol can be used, but it is commonly
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45 denatured with an unknown quantity of methanol, 2-propanol, hydrocarbons, or other
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47 compounds which might affect chromatography.
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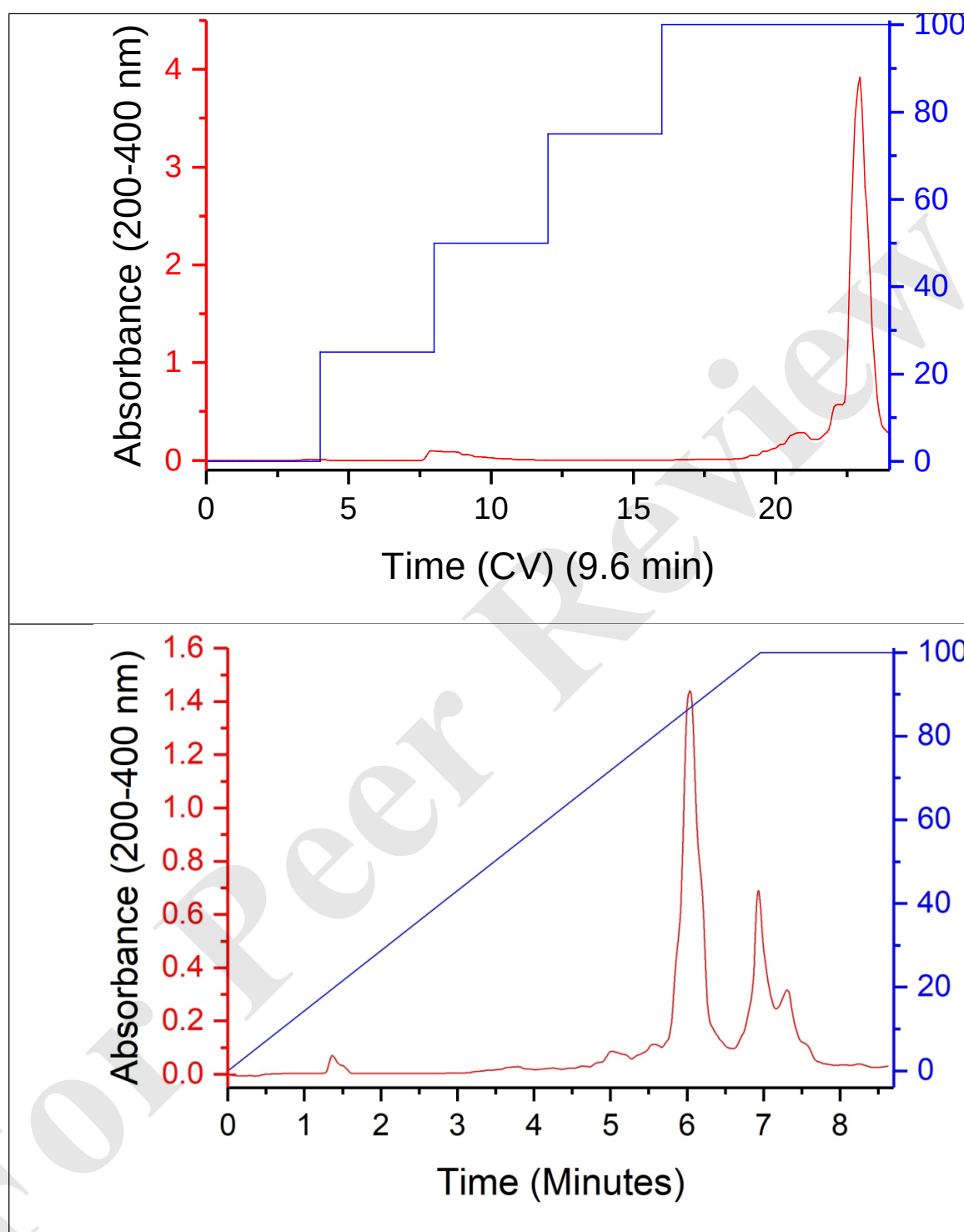
52 *Can we avoid chlorinated solvents altogether?*
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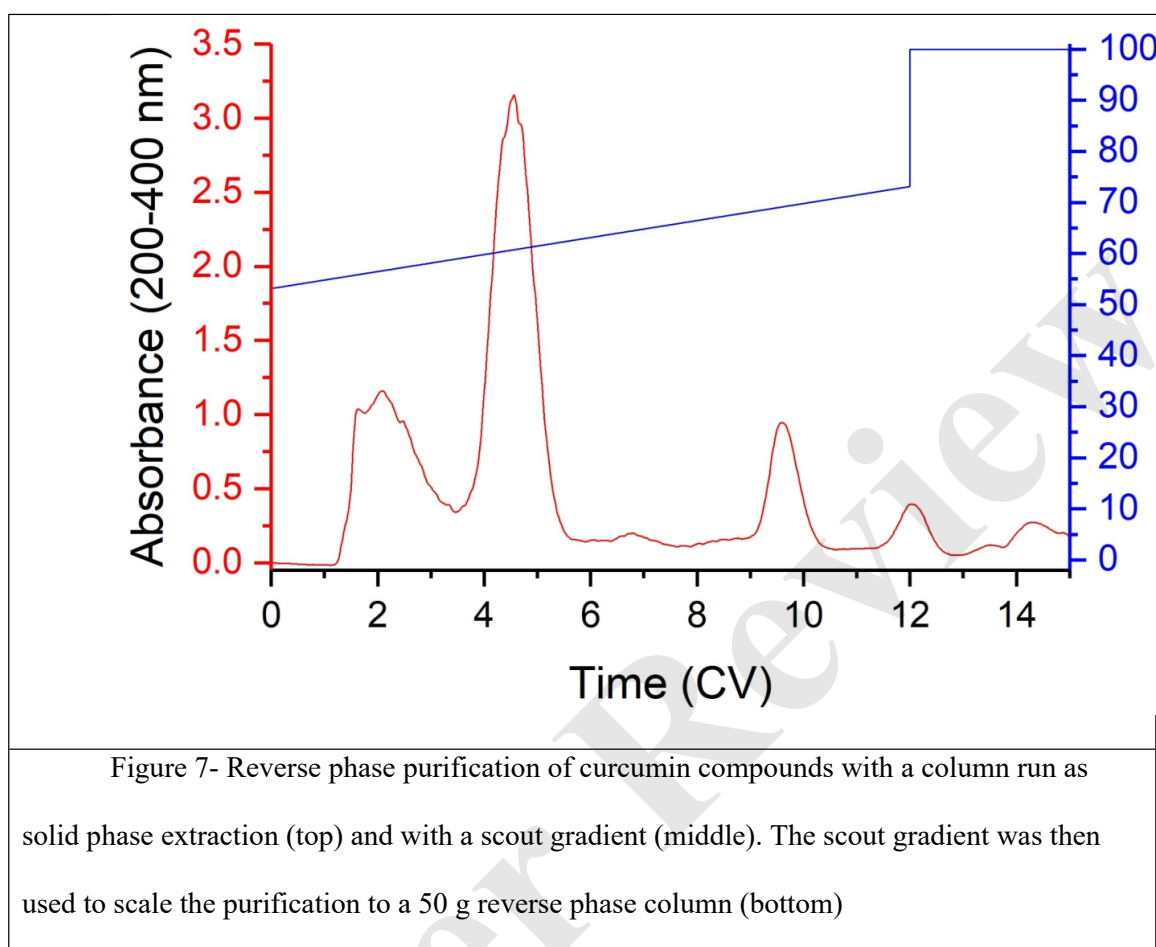
54 It may be possible to avoid chlorinated solvents during the entire purification process.
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56 Although the piperine (2) and curcumin (3,4,5) examples here used dichloromethane for
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4 the initial extractions, other solvents such as methanol, ethyl acetate, or acetone could
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6 be used instead.
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9 It is common to use silica as a first purification step after extraction, and
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11 chlorinated solvents are commonly used. Reverse phase flash chromatography can be
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13 used instead, and these columns are relatively inexpensive and reusable. In *Figure 7*, a
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15 flash scouting gradient is run on a 5.5 g flash column using methanol/water containing
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17 0.1% formic acid is used to calculate a purification method for a 50 g column, where
18
19 0.75 g sample was run. Also shown is a run with the 5.5 g column run as a solid phase
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21 extraction (SPE) cartridge. The step gradient employed for the SPE is the same as that
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23 described by Boudreau[27], except the steps were scaled to account for the column
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25 being slightly larger than the solid load cartridge. The scouting run allowed resolution
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27 of compounds and allowed a scale-up method to be determined. The scouting run used a
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29 similar amount of time and solvent but provided more information compared to the SPE
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31 experiment, allowing a purification method to be calculated. The scouting run removed
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33 more impurities which allows better LC-MS data or improved assay data.
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The peak eluting at 4.5 column volumes (CV) was used for the SFC runs described earlier. The reverse phase column is reusable, and no chlorinated solvents were required for the chromatography. Sephadex LH20 columns are also run as an early chromatography step and may be run without chlorinated solvents.

Conclusion

SFC is demonstrated to be a viable alternative to the use of chlorinated solvents to purify natural products. The references cited herein[10-12] listed 123 different studies of a range of non-polar and polar compounds purified using SFC; however, only one listed the use of DCM—fewer than 1% of the studies. The experiments run herein used compounds all known to be purified with chlorinated solvents with liquid

chromatography which were not required for SFC. As SFC is a normal phase technique, the guidelines for adjusting solvent composition based on solvent strength still apply.

Liquid chromatography can be run in such a way as to avoid chlorinated solvents, but there are many solvent systems to evaluate, whereas SFC needs only a few solvents to test. Although silica SFC may require additives, liquid chromatography also commonly uses solvent modifiers. It is noted that all the compounds run within this paper can be eluted from silica with an additive although the curcumin compounds needed a PEI column to resolve the different related compounds. This suggests that silica may be a first choice for SFC in the same role C18 plays for reverse phase liquid chromatography. Using the Time-on-Target algorithm with scouting runs allowed solvents and additives to be evaluated. Scouting gradients can be run on different columns as well. Note that so long as the analytical columns all have the same dimensions and use the same scouting gradient method, a single calibration will work for all of them with the Time-on-Target algorithm[17].

The purification of both piperine and curcumin compounds suggests that purification might be performed without chlorinated solvents, albeit with running reverse phase before the normal phase purification.

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Declaration of interest:

Jack Silver is an employee of Teledyne ISCO which manufactures and sell the preparative SFC, flash chromatography systems, and columns used in this paper.

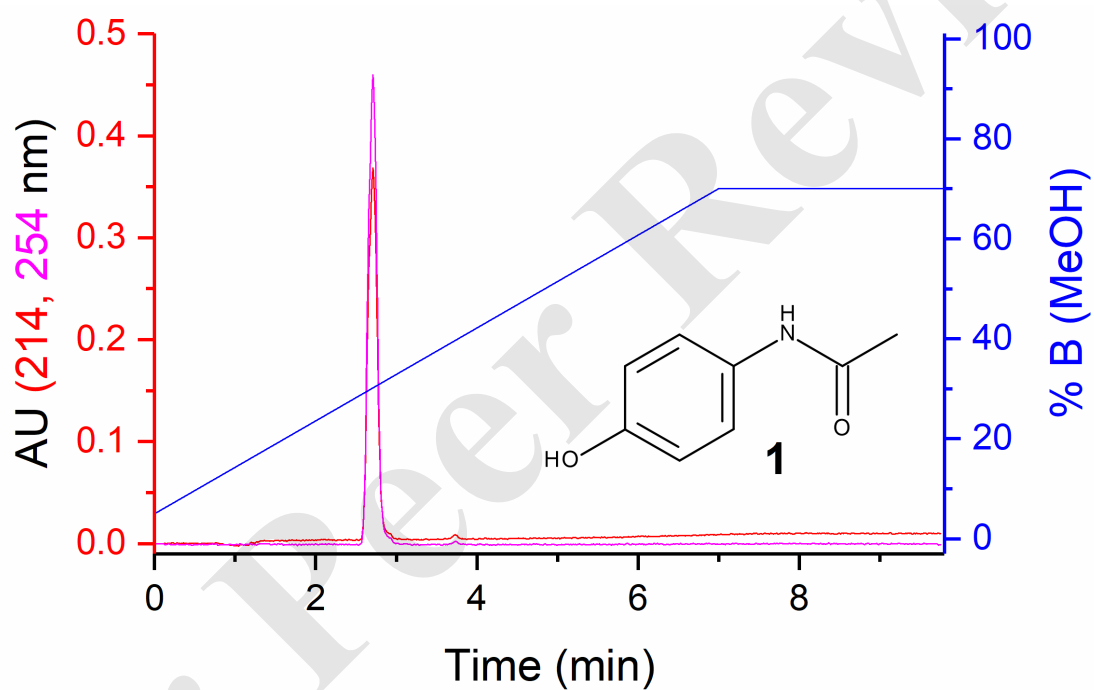
Huihui Xiao has no conflicts of interest.

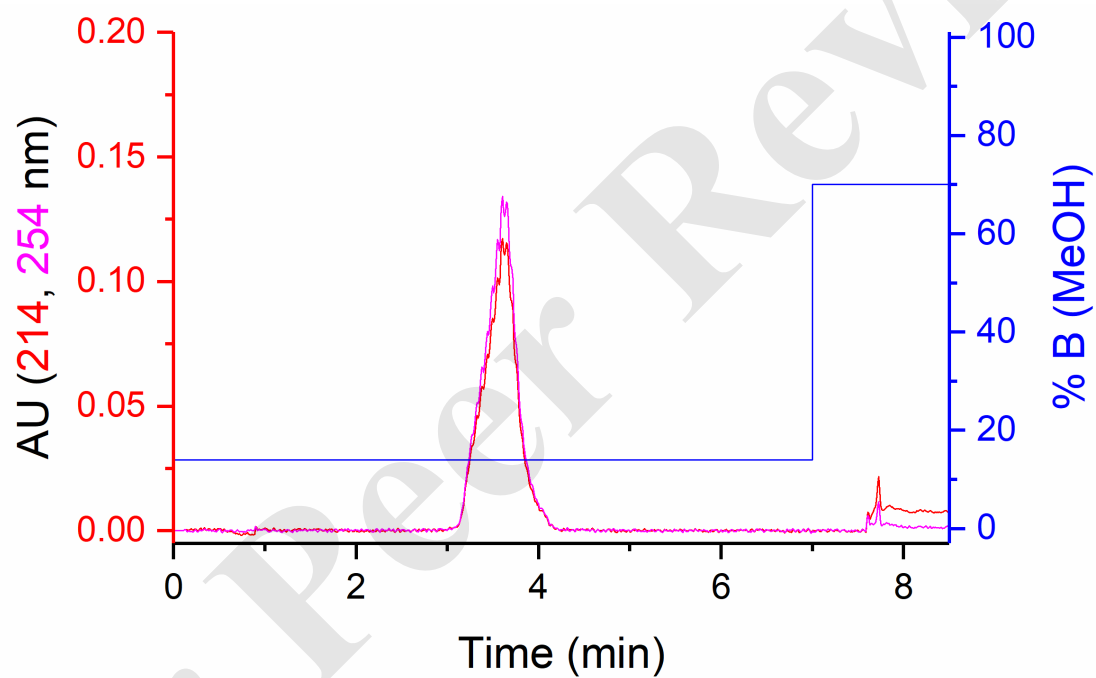
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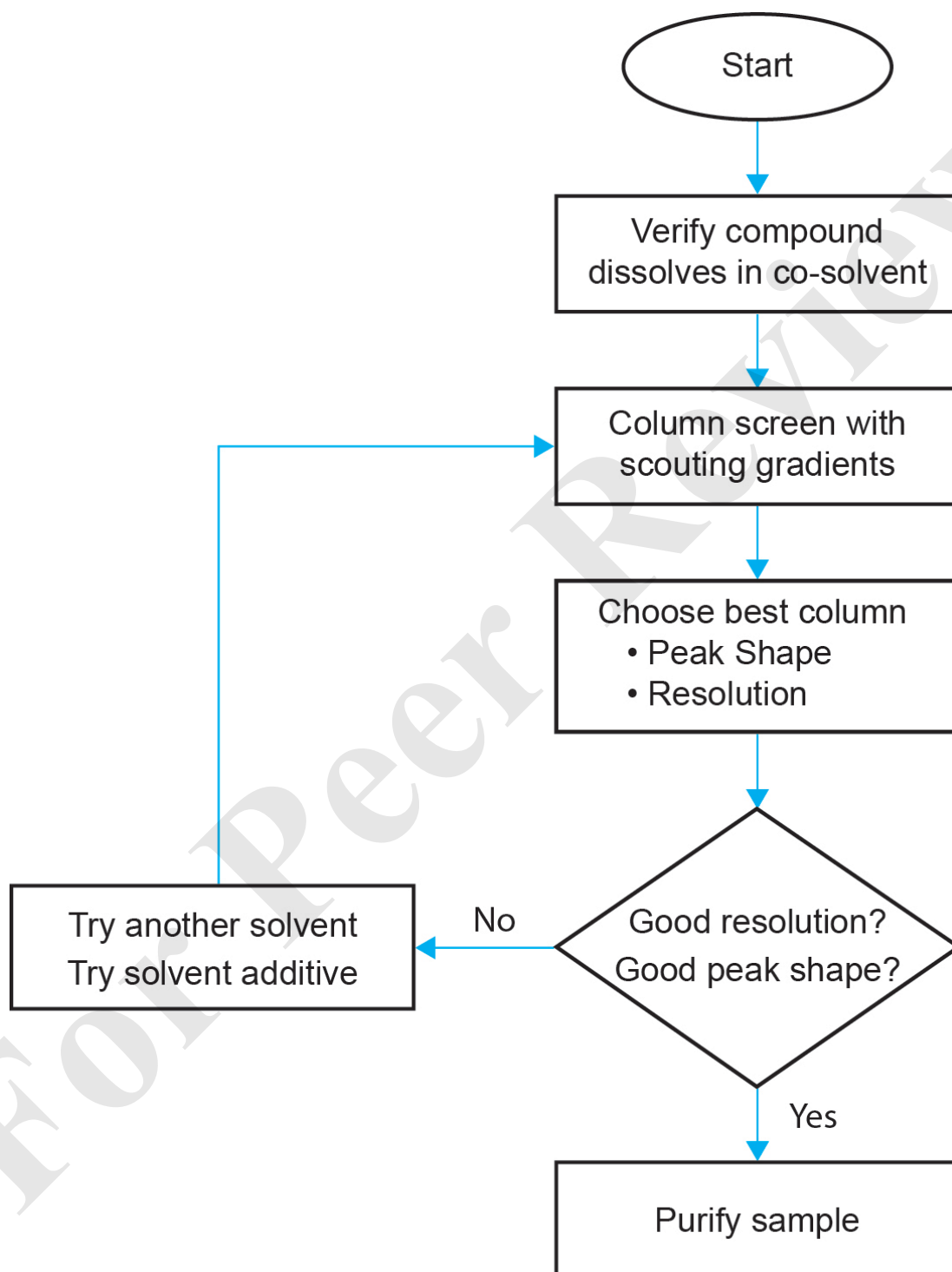
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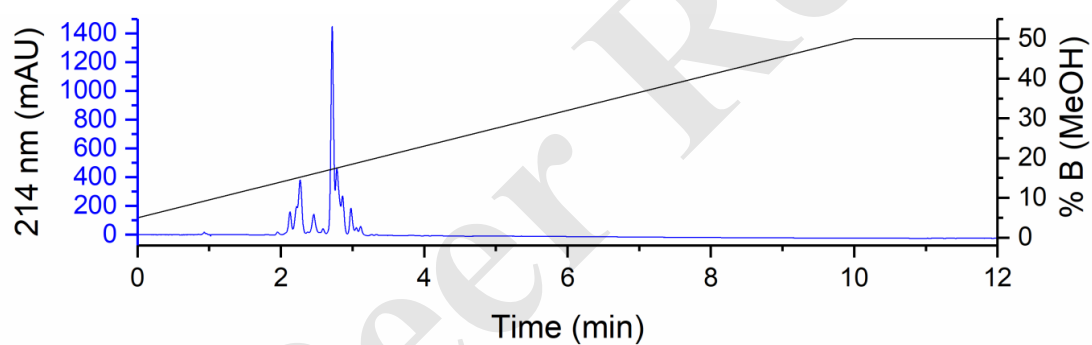
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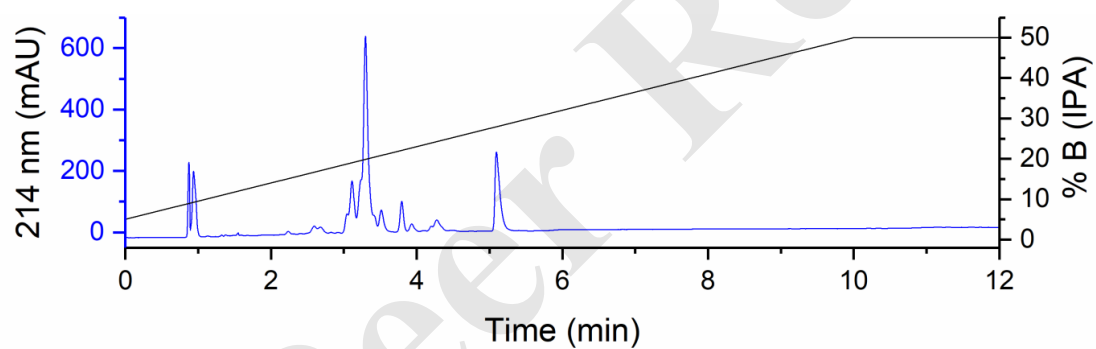


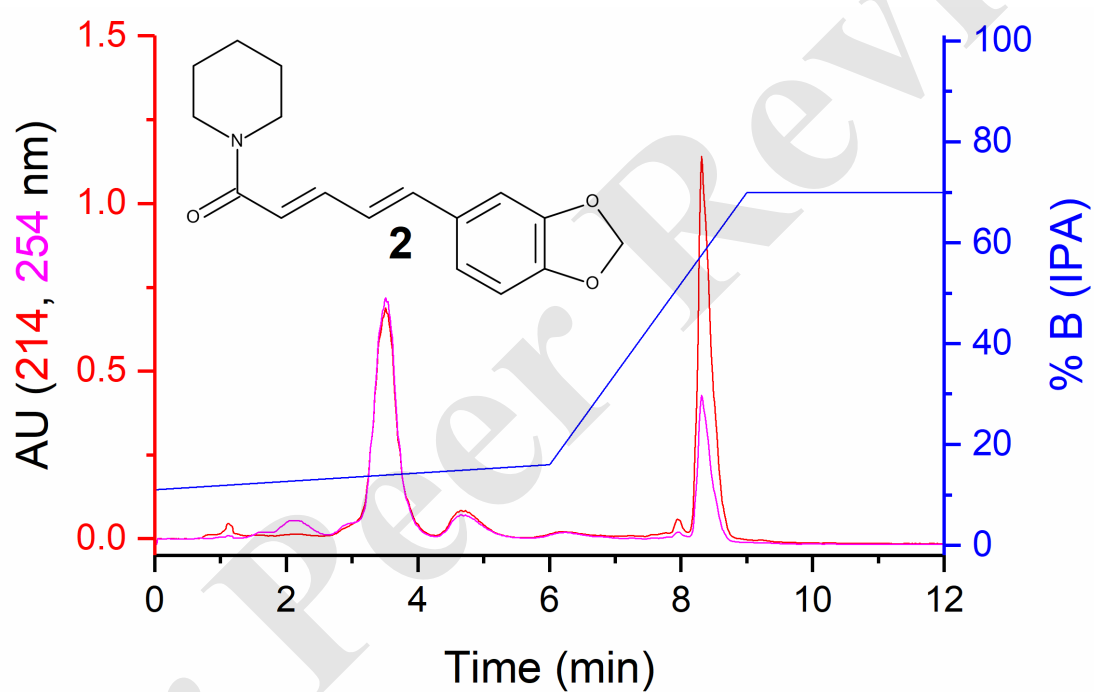


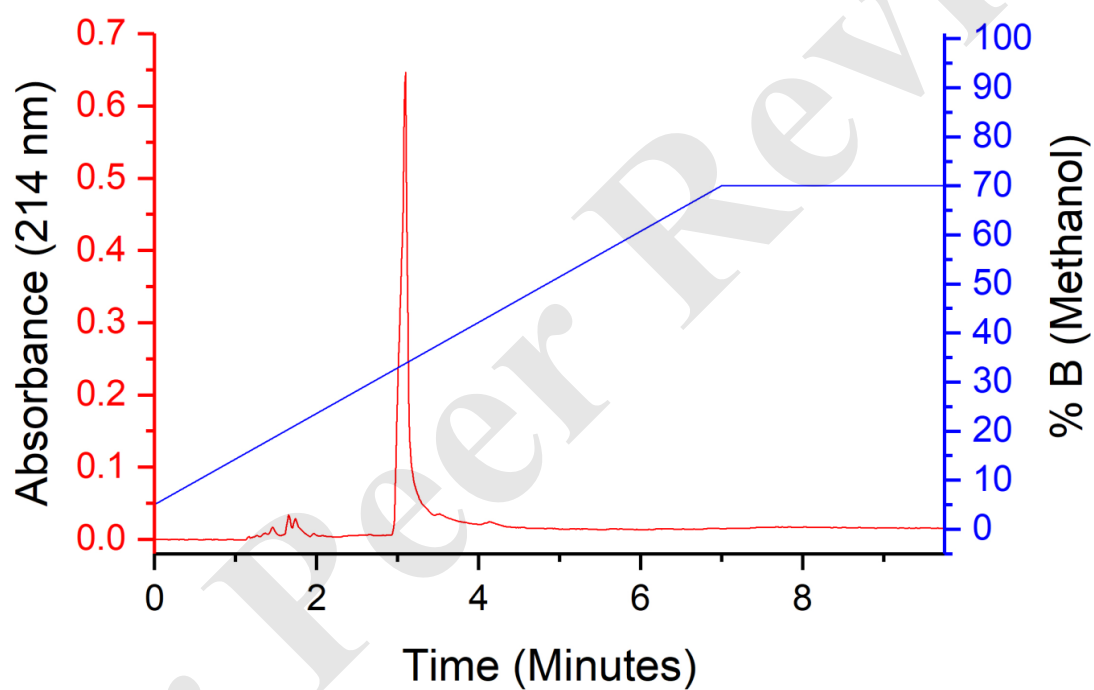


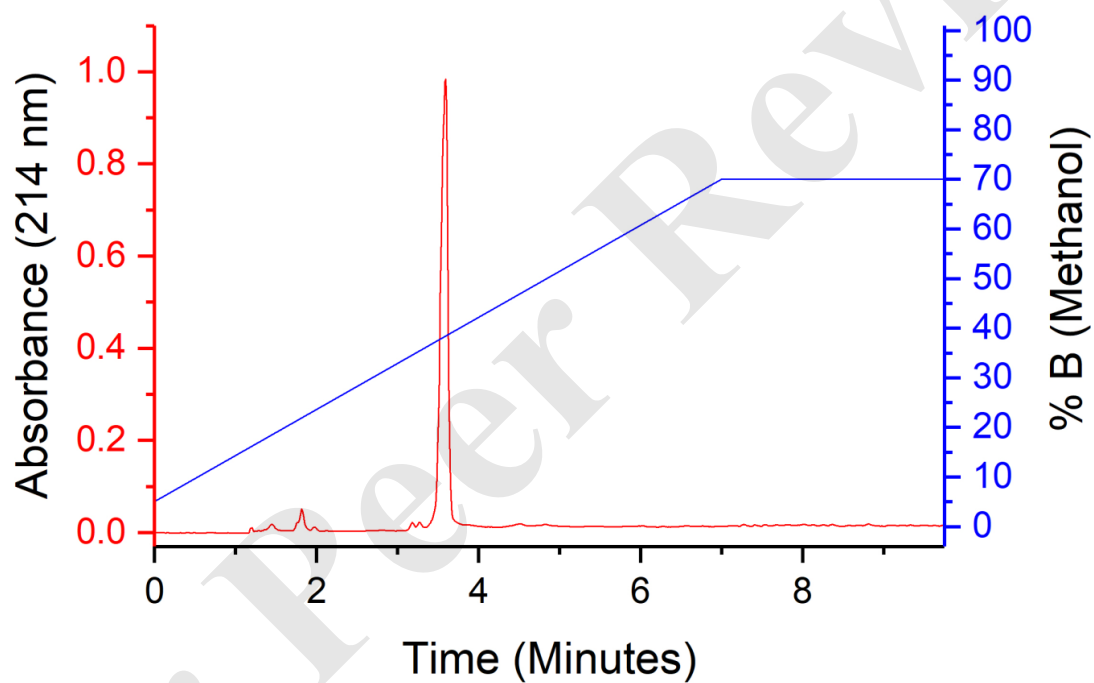


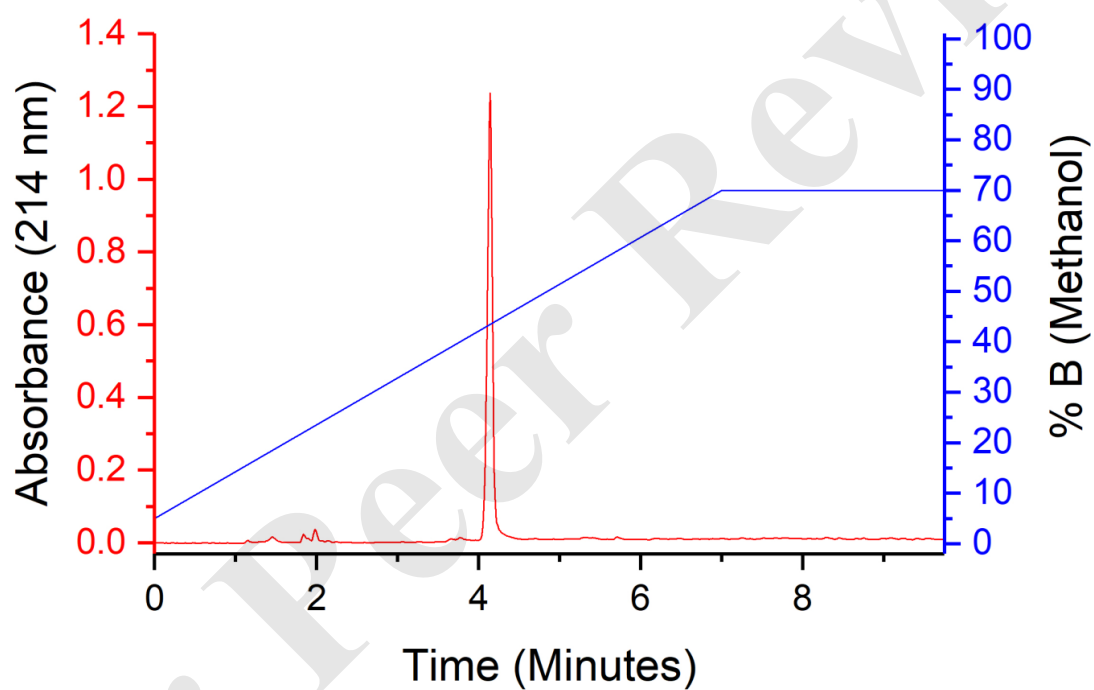


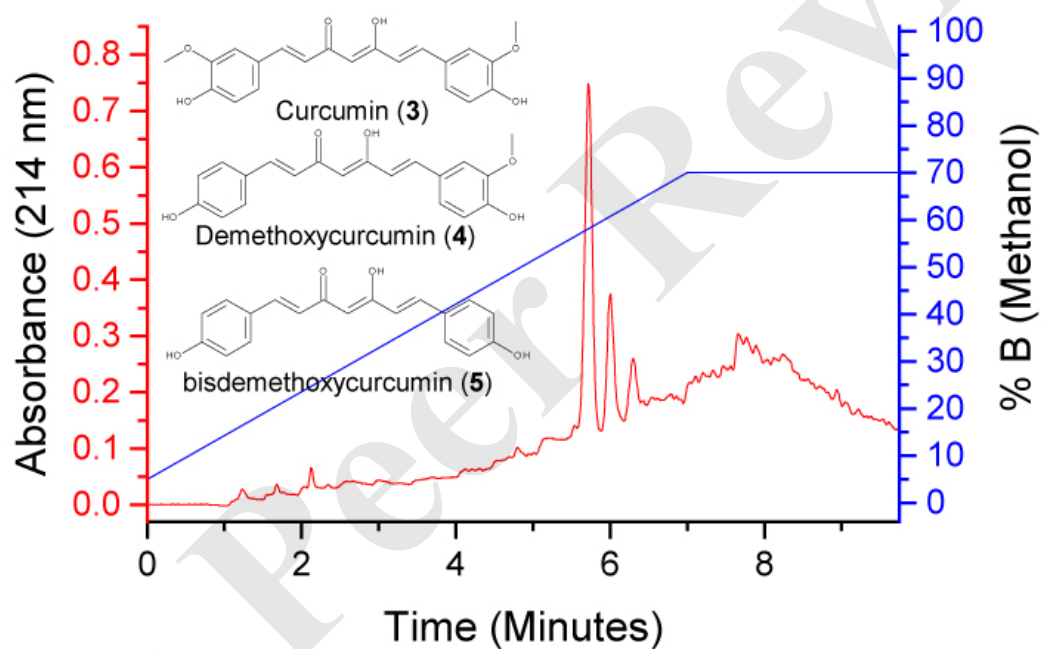


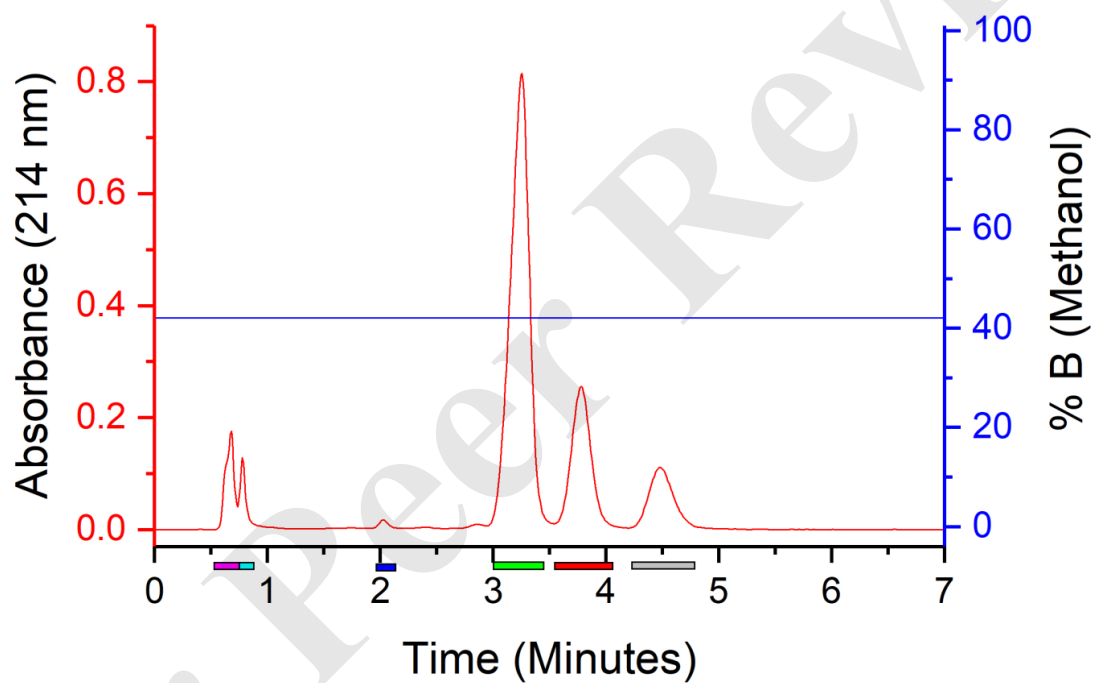


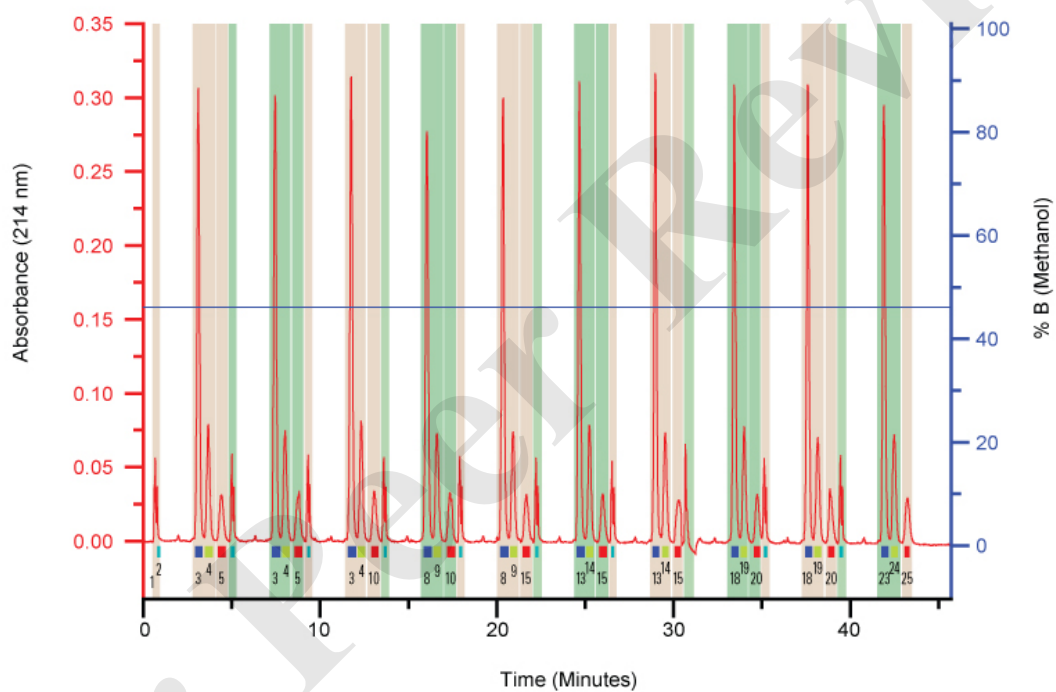


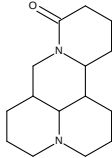
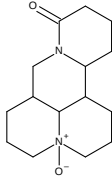


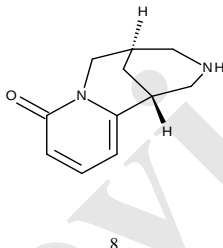
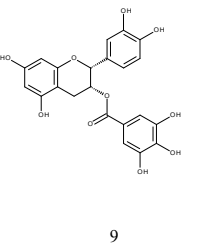


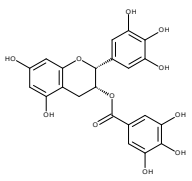
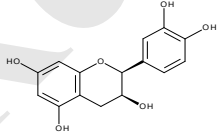






Compound[citation]	Column Focus gradient range	solvent	Chlorinated solvent used in original purification	Structure
matrine[25]	Silica Broad peak in scout run	methanol, no additive	dichloromethane with methanol	 6
	Diol Broad peak in scout run	methanol, no additive		
	4-ethylpyridine 18.1-28.1	methanol, no additive		
	PEI 26.6-36.6	methanol, no additive		
	Silica 30.7-40.7	methanol 0.5% isopropyl amine		
oxymatrine[25]	Silica No elution	methanol	dichloromethane with methanol	 7
	Diol Late elution, severe tailing	methanol		
	4-ethylpyridine 34.3- 44.3	methanol		
	PEI	methanol		

	Some tailing			
	Silica 58.0-68.0	methanol 0.5% isopropyl amine		
cytisine[25]	Silica- very broad peak	methanol	dichloromethane with methanol	
	Diol Broad peak	methanol		
	4-ethyl pyridine 17.5 – 27.5	methanol		
	PEI 26.7-36.7	methanol		
	Silica 28.4-38.4	methanol 0.5% isopropyl amine		
(-)-Epicatechin 3- gallate[26]	Silica 24.7 -34.7	methanol	dichloromethane with methanol	
	Diol Peak tailed badly	methanol		

(-)-Epigallocatechin 3-gallate[26]	Silica Wide, tailing peak in scout	methanol	dichloromethane with methanol	 10
	Diol 38.4-48.4	methanol		
	Silica 25.8-38.8	Methanol 0.5% FA		
Epicatechin	Silica 19.0- 29.0	methanol	This is found with other catechins and was run to demonstrate it could be resolved.	 11
	Silica 18.2-28.2	methanol 0.5% FA		
Table 1- compounds purified via column chromatography using chlorinated solvents and run on SFC. Compounds showing acceptable peak shape for a column and co-solvent have a focused gradient range listed.				

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