

Purification of natural products

Case study 1: *Scutellaria baicalensis*

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Overview

According to a survey published by the *Journal of Natural Products* in 2003, a majority of small molecules reaching clinical study stage over the last two decades of the twentieth century can be traced to natural products. In some therapeutic areas such as antibacterial and anticancer areas, natural products are the dominant source of successful new bioactive compounds.

The purification of natural products remains challenging, lengthy, and tedious. Automated flash chromatography instruments ease this process. This application note provides a suggested general methodology for natural products purification and an illustration of this methodology using Teledyne Isco's CombiFlash® instruments and RediSep® columns.

General Procedure

The suggested procedure for natural products purification breaks down to the following three main steps:

1. Collect general analytical information.

Initial general information gathering about the natural product sample to be purified is useful to obtain a qualitative overview about the sample's composition and complexity.

- A gas chromatography (GC) analysis provides the number of compounds expected in the sample.
- A UV spectrophotometer scan provides the optimal absorbance wavelength(s) for the sample under investigation.
- A C-18 reversed phase¹ TLC plate eluted with H₂O/CH₃CN 1:1 and then exposed to UV light provides general information.
- Initial small-scale chromatogram using:
 - a 20mg sample with a 4.3g RediSep C-18 reversed phase column
 - the optimal wavelength(s) previously found in step 1b.
 - a default gradient elution 0–100% acetonitrile in H₂O.

2. Separate the full batch under conditions determined from step 1.

The total batch of sample undergoes a bulk purification at this stage.

- Select the appropriate size C-18 reversed phase RediSep column to meet a ~0.5% w/w sample/column size loading capacity.
- Elute the products using a gradient solvent system determined by the observed products' polarities in the small sample run(s) from step 1.

The resulting single peaks which can be fully isolated at this stage do not need further action.

3. Separate resulting pockets of unresolved peaks obtained from step 2.

Collect unresolved peak group fractions and prepare them for subsequent purification. Gradient elution needs precise tailoring to separate each compound located in these groups.

Background

To illustrate the application of this suggested procedure, a purification of *Scutellaria baicalensis* was investigated.

Scutellaria baicalensis, commonly known as Chinese scullcap or Baikal scullcap, is a member of the mint family, grown in China and Russia. The root of this plant is used in traditional Chinese herbal medicines. This scullcap variety has been the focus of many studies because of its historical use in treating inflammatory skin conditions, bronchitis, hay fever, and hepatitis.

Test tube studies have documented that the root of Chinese scullcap inhibits bacteria and viruses, and promotes anti-allergy actions. This root also contains the flavonoid substance baicalin, which has received much attention in therapeutic studies. Rudimentary human trials suggest that Chinese scullcap may aid the treatment of acute lung, intestinal, and liver infections, as well as hypertension.

1. Natural products contain numerous compounds with a wide range of polarities, a media which can potentially migrate them all should be considered. Reversed phase silica gel meets this requirement and is the suggested media for Thin-layer Chromatography (TLC) plates and columns runs.

Results and Discussion

A *Scutellaria baicalensis* sample purification follows the general procedure suggested earlier:

1. Collect general analytical information.

The GC analysis showed that the natural product contains six compounds (Figure 1).

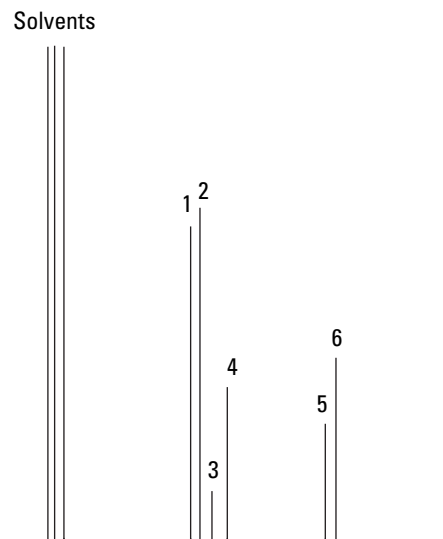


Figure 1: *Scutellaria baicalensis* GC analysis

The C-18 reversed phase TLC plate run (Figure 2) provided another visualization of the natural product sample composition. The number of products identified on GC was confirmed by TLC.

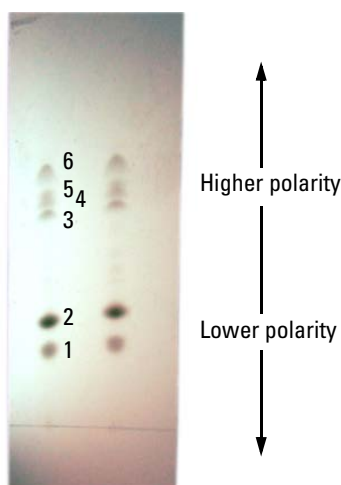


Figure 2: *Scutellaria baicalensis* C-18 Reversed phase TLC plate Solvent system: H₂O/CH₃CN, 1:1

The polarity of each component can be noted from TLC which may be of assistance when designing gradients at a later stage. Generally, a C-18 reversed phase TLC plate eluted with H₂O/CH₃CN 1:1 migrates the least polar compounds to the lower half of the TLC plate and

the most polar compounds to the upper half of the TLC plate. *Scutellaria baicalensis* contains four medium to high polarity compounds that migrated to the upper half of the plate and two medium-low polarity compounds that migrated to the bottom half of the plate.

The UV spectrophotometer scan (Figure 3) suggested an optimal absorption at a wavelength of 277 nm. Several trial purifications at different wavelengths confirmed that 277 nm provides the best absorption and sharper peaks.

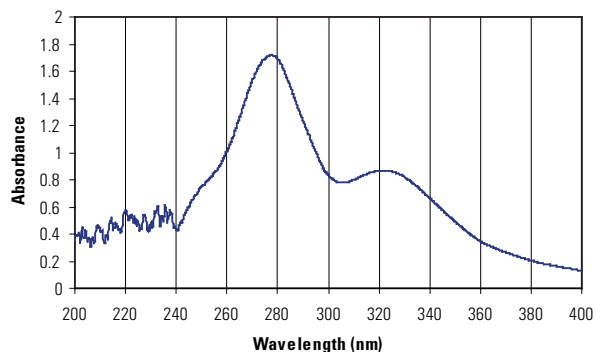


Figure 3: *Scutellaria baicalensis* sample UV spectrophotometer scan $\lambda_{\text{max}} = 277 \text{ nm}$

An initial chromatogram was obtained with a small natural product sample (Figure 4 and Table 1) at the optimal wavelength.

This initial chromatogram showed a clear separation of the two least polar compounds eluting at Column Volume (CV) =33 and CV=37. On a larger-scale purification run, these two compounds would be expected to easily separate using a simple linear gradient solvents elution.

The peak group including several rather polar compounds eluting between CV=25 and CV=30 will likely need to go through an additional purification process with revised parameters to increase resolution.

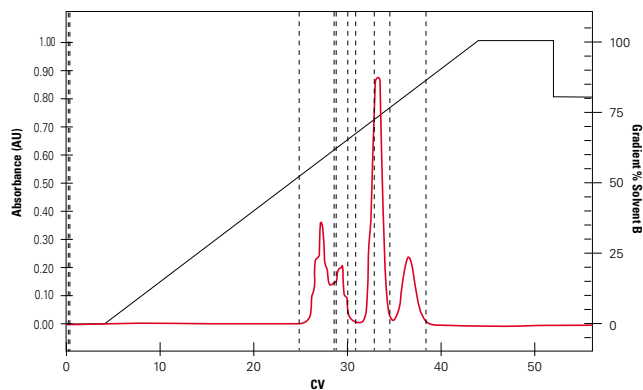


Figure 4: Small scale natural product initial purification chromatogram

2. Separate the full batch under conditions determined from step 1.

The natural product full batch purification was undertaken. Information gathered during step 1 of the process indicated that the polar peak group eluting first contained several compounds with close polarities. This group requires an isocratic plateau to improve resolution of the discrete compounds. It is observed in Figure 4 that the peak group starts eluting at ~50% acetonitrile. Dividing this percentage of solvent B by two generally provides a %B value for an isocratic plateau that will partially or fully resolve the grouped peaks. Therefore a gradient plateau at 25% acetonitrile over 15 column volumes initiated the gradient elution for the full batch separation.

The information gathered from step 1 also indicated straightforward separation of the two least polar large peaks. Therefore the second part of the gradient elution followed a linear gradient pattern. The gradient ended with a final column wash with 100% acetonitrile followed by a plateau of 80% acetonitrile without an air purge. This wash and finish with 80% acetonitrile leaves the C-18 reverse phase column ready for reuse.

The natural product full batch run at 277 nm following the elution pattern just discussed (Figure 5 and Table 2) provided separation of the initial large peak group into two smaller peak groups. Each of these groups apparently contained two compounds. The major two medium-low polarity products were fully isolated.

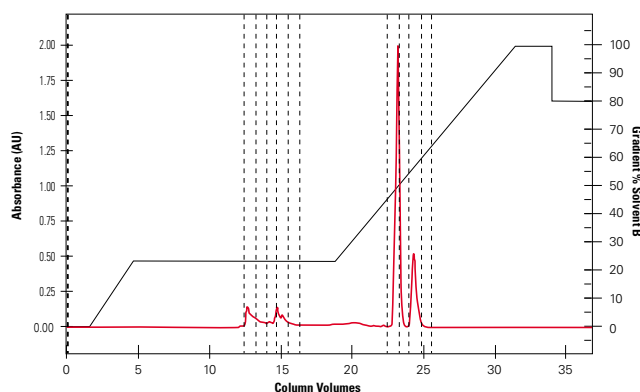


Figure 5: Full batch natural product purification chromatogram

3. Separating resulting pockets of unresolved peaks obtained from step 2.

The collected fractions for each unresolved peak groups observed in step 2 are gathered and concentrated for further purification.

The first peak group (Figure 5, CV=12) contains two rather polar products. To achieve a satisfactory separation of compounds with rather polar properties, starting the gradient with the least polar solvent is often a practical approach.

The separation of the first peak group was achieved starting the gradient elution with a 50% acetonitrile solvent system (Figure 6 and Table 3). Two products were isolated and collected.

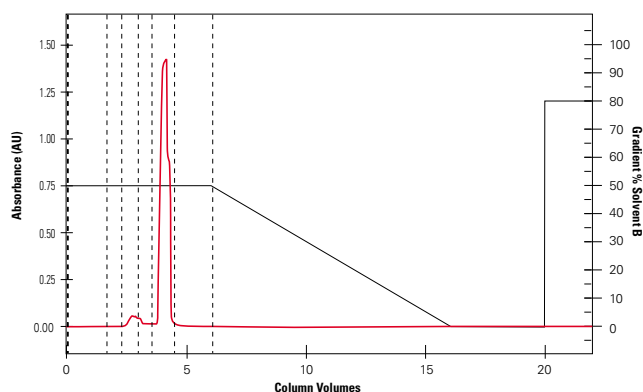


Figure 6: First peak group purification chromatogram

Similarly, the second peak group (Figure 5, CV=15) also contained two rather polar products. The same approach was taken, starting the gradient elution with a 50% acetonitrile solvent system (Figure 7 and Table 4). Two products were isolated and collected.

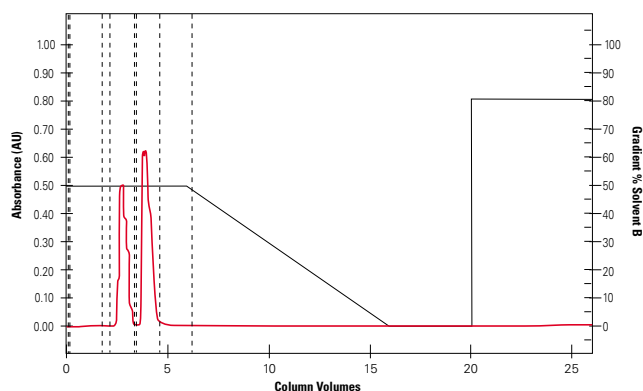


Figure 7: Second peak group purification chromatogram

In summary, the six compounds initially counted by GC and TLC in *Scutellaria baicalensis* were all isolated via a progressive flash chromatography purification procedure using C-18 reversed phase RediSep columns with tailored gradient elutions.

Experimental

Table 1: Method Parameters

Instrumentation:	Teledyne Isco CombiFlash [®] Companion™ 4x	
Column	4.3 g C-18 Reversed Phase RediSep	
Sample Loading Method	23 mg pre-loaded on C-18 reversed phase silica powder	
Wavelength	277 nm	
Mobile phase:	Solvent A: Water	Solvent B: Acetonitrile
Flow Rate:	12 mL/minute	
Equilibration Volume:	7 column volumes	
Gradient:	% Solvent B	CV
	0	Initial
	0	4.0
	100	40.0
	100	8.0
	80	0.0
	80	4.0

Table 2: Method Parameters

Instrumentation:	Teledyne Isco CombiFlash [®] Companion™ 4x	
Column	130 g C-18 Reversed Phase RediSep	
Sample Loading Method	841 mg pre-loaded on C-18 reversed phase silica powder	
Wavelength	277 nm	
Mobile phase:	Solvent A: Water	Solvent B: Acetonitrile
Flow Rate:	50 mL/minute	
Equilibration Volume:	7 column volumes	
Gradient:	% Solvent B	CV
	0	Initial
	0	2.0
	25	2.0
	25	15.0
	100	12.0
	100	3.0
	80	0.0
	80	3.0

Table 3: Method Parameters

Instrumentation:	Teledyne Isco CombiFlash [®] Companion™ 4x	
Column	13 g C-18 Reversed Phase RediSep	
Sample Loading Method	78 mg pre-loaded on C-18 reversed phase silica powder	
Wavelength	277 nm	
Mobile phase:	Solvent A: Water	Solvent B: Acetonitrile
Flow Rate:	15 mL/minute	
Equilibration Volume:	7 column volumes	
Gradient:	% Solvent B	CV
	50	Initial
	50	6.0
	0	10.0
	0	4.0
	80	0.0
	80	2.0

Table 4: Method Parameters

Instrumentation:	Teledyne Isco CombiFlash [®] Companion™ 4x	
Column	13 g C-18 Reversed Phase RediSep	
Sample Loading Method	83 mg pre-loaded on C-18 reversed phase silica powder	
Wavelength	277 nm	
Mobile phase:	Solvent A: Water	Solvent B: Acetonitrile
Flow Rate:	15 mL/minute	
Equilibration Volume:	7 column volumes	
Gradient:	% Solvent B	CV
	50	Initial
	50	6.0
	0	10.0
	0	4.0
	80	0.0
	80	2.0

Conclusion

A general procedure for natural product purification was proposed.

Following this general procedure, the purification of a natural product, *Scutellaria baicalensis*, was successfully achieved using adequately Teledyne Isco's CombiFlash instruments and RediSep columns.

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