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INTRODUCTION

In the 1990s, the pace of drug discovery accelerated rapidly as screening and chemical synthesis transitioned from the traditional relatively linear iterative process to parallel approaches. High-speed parallel synthesis created the need for rapid analysis and screening by HPLC. Beginning about 1996, the use of reversed phase gradients on C₁₈ media in short column formats as 'generic separation methods' developed in critical applications such as analysis of crude synthetic isolates, in-vitro drug metabolism assays, and purification of drug discovery leads.

Reversed-phase gradient HPLC is now frequently used for purification. A common approach is to inject up to 100 mg of crude synthesis product on a 20 x 50 mm 5 μ C₁₈ column and elute with a <10 minute steep gradient of water and acetonitrile, usually with an organic acid or buffer modifier. MS detection (or UV detection after LCMS analysis) ensures that the desired peak is collected. A disadvantage is the slow and warm dry-down of fractions necessitated by the aqueous media.

Might normal phase chromatography, HPLC and SFC, be adapted to allow their use with 'generic' separation methods? What are the characteristics of a successful 'generic' method? What hurdles must be overcome if SFC is to be as generally useful as RP-HPLC?

DEFINING 'GENERIC' GRADIENTS

An *optimized* method disperses analytes and artifacts into separate bands that elute with predictable profile and retention time and evoke a response at a detector proportional to the quantity of compound. A *generic* method is used without optimization – every analyte or mixture is applied to the column without regard to whether the detector will respond or saturate; without consideration of whether mixture components will co-elute. Time constraints make traditional method optimization impossible; but more importantly, compound-specific optimization is not desirable: efficient drug discovery aims to understand structure-activity relationships across large sets of compounds, and this task is simplest when each is handled identically.

Generic chromatography is a practical art. Definitions of good chromatography – analytical figures of merit used to evaluate the strength of an optimized method – largely do not apply. Instead, *the only valid measure of good is whether the job at hand gets done* – is the desired compound distinct within the sample injected?

Table 1. Attributes of Generic Gradients & Enabling Technologies

Attribute	Enabling Technology
Retention and elution of most compounds of interest: High peak capacity	Gradient elution HPLC solid phase characteristics: – Small particles, high phase loading – End-capping – pH and water-stable phases
Universal detection	Diode array UV detection Mass Spectrometry (MS) detection Evaporative Light Scattering Detection (ELSD) Chemiluminescent Nitrogen Detection (CLND)
Differential (specific) detection	Diode array UV detection MS and MS/MS detection
Rapid chromatographic cycle	Short, efficient columns Alternating column regeneration Low dwell volume HPLC Ultra High Pressure LC (UPLC)

Without the development of three primary technologies, gradient HPLC would not have developed as a generic analytical approach:

- Generally retentive phases, such as C₁₈
- Atmospheric pressure (API) mass spectrometric detection
- Short, high capacity HPLC columns

In other words, it was necessary to demonstrate that the majority compounds of pharmaceutical interest could be retained and eluted from the same column, and that one could detect the single component of interest selectively. It was also necessary to do the analysis rapidly enough that large compound sets could be analyzed, as the pressure to develop generic methods was coming from the parallel chemistry movement.

Prior to 1997, few columns were available with small particle size and in shorter formats. The ubiquity of rapid generic gradients with RP-HPLC drove the development of columns, phases, and systems technology rapidly over the last 10 years.

The lopsided development of RP-HPLC over normal phase and SFC in generic gradient applications is partially due to the fact that ESI-MS sources, until recently, required a mixed aqueous solvent to generate [M+H]⁺ ions. Recent developments in API MS sources have enhanced sensitivity and allow analytes to be ionized successfully from virtually any mixed solvent system containing a proton donor (e.g. hexane/methanol, CO₂/methanol). Recently, commercially available SFC-MS systems have demonstrated not only easy coupling but highly stable and reliable ionization of pharmaceutical compounds.

COLUMN SELECTIVITY

Using a mixture of drugs and drug-like like compounds as a test standard, we examined a variety of stationary phases for compound retention and elution.

In evaluating the chromatograms, we are interested in whether all compounds are retained and elute, a necessary condition in a generic method. We took note of broadened or tailing peaks, an indication of incomplete transfer to the stationary phase and of inefficient mass loading. Loading is critical if preparative chromatographic methods are to be developed.

Table 2. Drug-Like Compounds Used in the Test Standard

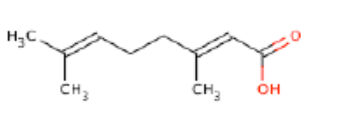
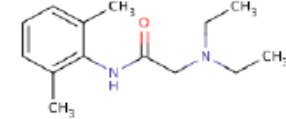
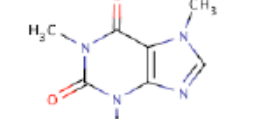
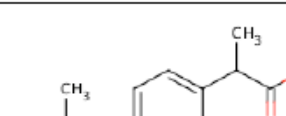
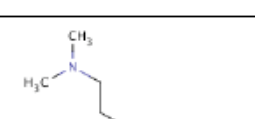
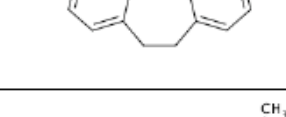
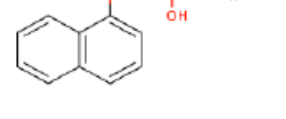
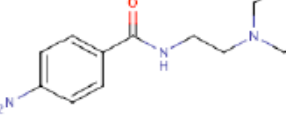
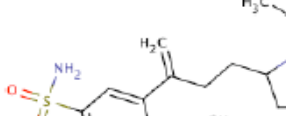

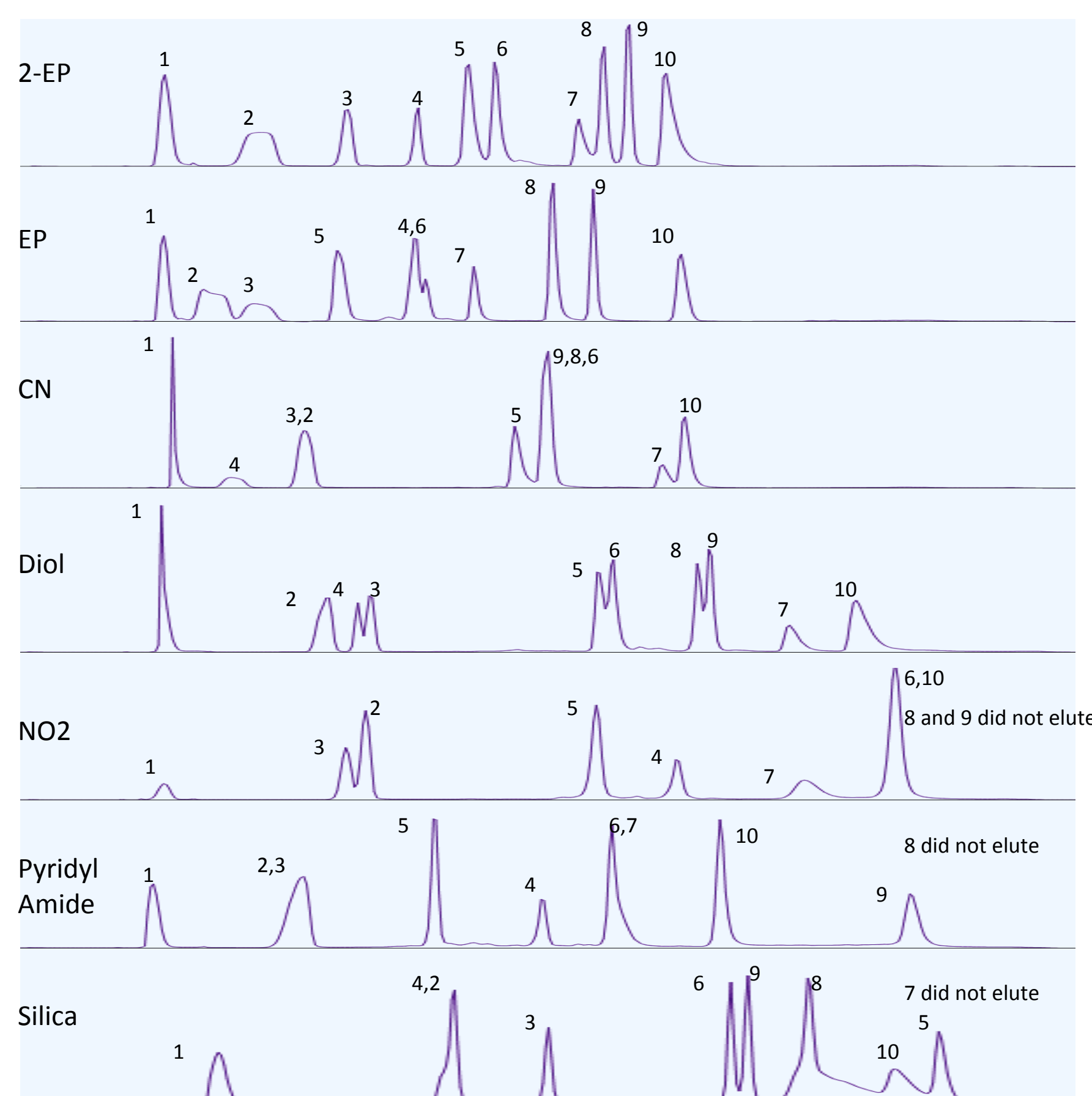
Compound (elution order on 2-EP)	Name	
1	Citral	
2	Lidocaine	
3	Caffeine	
4	Ibuprofen	
5	Amitriptyline	
6	Propranolol	
7	Procainamide	
8	Sulpiride	
9	b-D-Arabinofuranosyl-cytosine	
10	Sulfinpyrazone	

Table 3. Initial Selection of Study Columns Used With Standard Elution Method

Column	Description
2-EP	2-Ethylpyridine 5 μ , 4.6x100mm, Princeton Chromatography
EP	Chromegabond Ethylpyridine SFC 5 μ , 4.6x100mm, ES Industries
CN	PrincetonSFC CN, 5 μ , 4.6x100mm, Princeton Chromatography
Diol	PrincetonSFC Diol, 5 μ , 4.6x100mm, Princeton Chromatography
NO2	Chromegabond Epic-NO2 SFC, 5 μ , 4.6x100mm, ES Industries
Pyridyl Amide	Chromegabond Pyridyl Amide SFC, 5 μ , 4.6x100mm, ES Industries
Silica	PrincetonSFC Silica, 5 μ , 4.6x100mm, Princeton Chromatography

The test standard was injected onto each column using an SFC gradient method of 5%-65% cosolvent in CO₂ (total flow of 2.0 mL/min) over 5 minutes, followed with a 10 second hold at 65% and a return to initial condition. The chromatograms shown in Figure 1 are presented on an 8 minute x-axis. The mobile phase cosolvent is 50:50 methanol:isopropanol with 0.1% diethylamine (MeOH:IPA 0.1%DEA).

Figure 1. Column Selectivity Study Chromatograms



Note that retention and peak shape on unmodified silica is worst. Unmodified silica is a poor choice for generic chromatography due to its stability and reactivity. The bonded phases, while showing large differences in selectivity, offer a range of alternatives in the application.

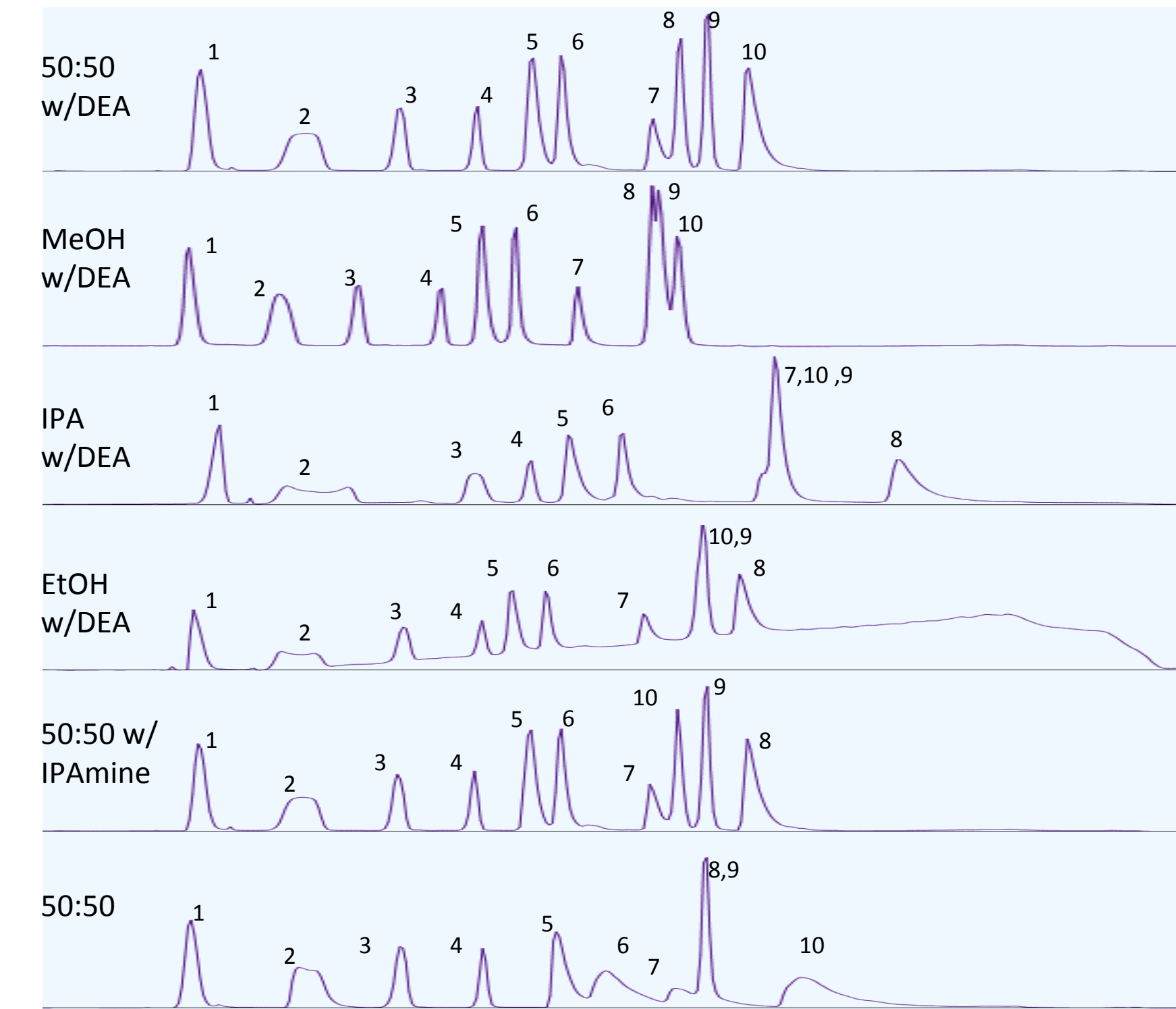
SOLVENT SELECTIVITY

Choosing - somewhat arbitrarily - the 2-EP phase, we compared its performance with the test standard using a selection of common SFC cosolvents. These are shown in Table 4, and the results in Figure 2.

Table 4. Solvent Abbreviations Used in Figure 2

Solvent	Description
50:50	50% methanol in 2-propanol
MeOH	Methanol
EtOH	Ethanol
IPA	2-Propanol
DEA	Diethylamine additive at 0.1% v/v
IPAmine	2-Propylamine additive at 0.1% v/v

Figure 2. Solvent Selectivity Study Chromatograms



Although 2-EP has a reputation for producing better peak shape with moderately basic analytes than other (less basic) bonded phases, the cosolvent with no organic base modifier allows substantial tailing with propranolol, procainamide, and sulfinpyrazone. We have no estimate of these compounds pKa values in mixed organic solvent, but believe the tailing is related to their relative basicity.

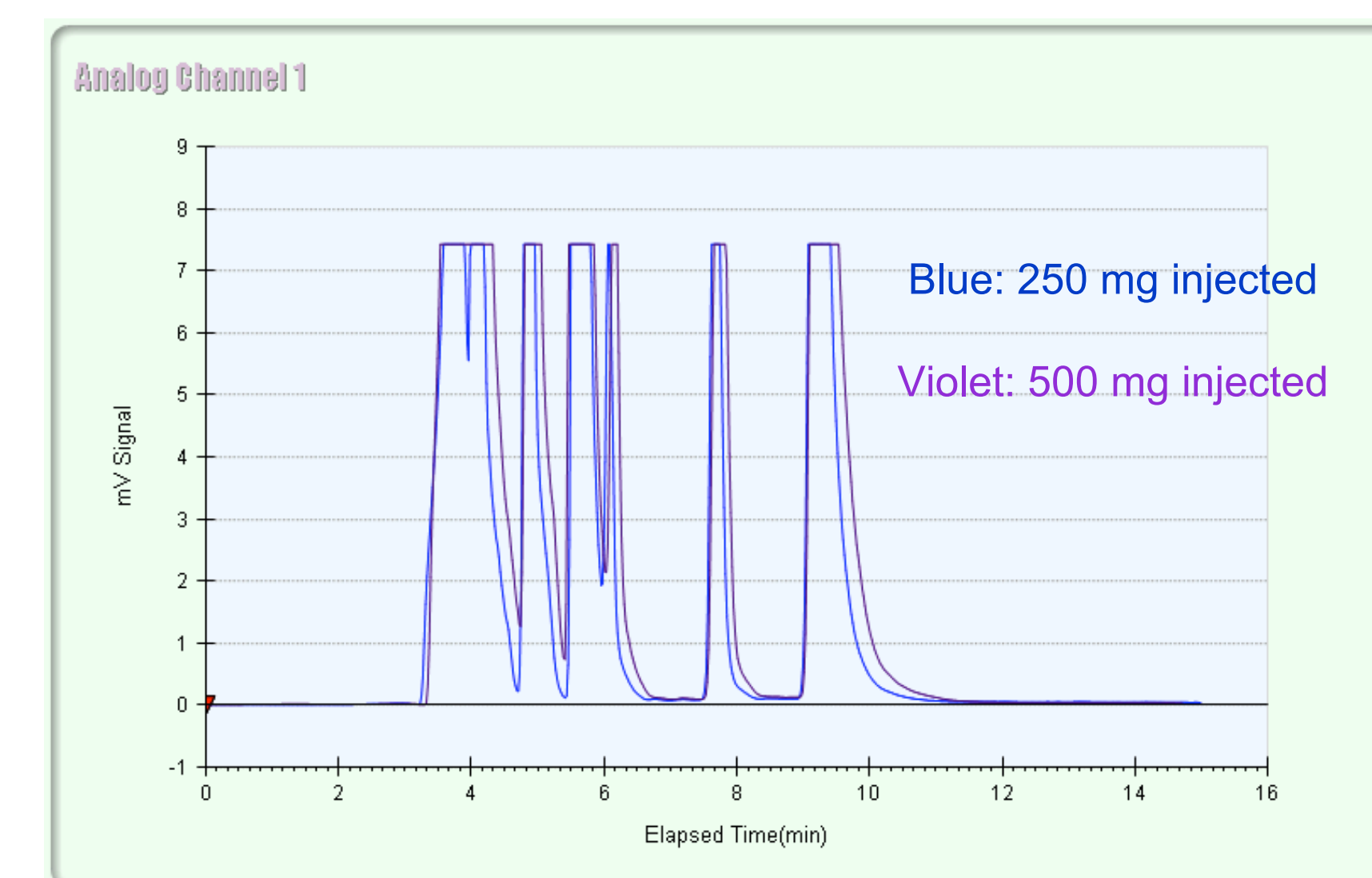
We note substantial differences in selectivity of the column across the solvent series MeOH/50:50/IPA. However, despite changes in elution order all the solvent systems meet the basic criteria of an adequate generic separation method.

Note that in the applications of generic RP-HPLC, chromatographers frequently screen unknown mixtures using two methods in which the same column is used with two solvent systems offering different selectivity. For example, amines may be retained on C₁₈ using a water/acetonitrile/ammonium bicarbonate buffer, while other compounds are better retained and resolved using water/acetonitrile/formic acid. We suggest that a similar approach, a standard column run with two methods offering different analyte selectivity, might be a useful approach with generic SFC.

PREPARATIVE GENERIC GRADIENT SFC

Figure 3 shows the result of loading a five compound mixture (procainamide, sulpiride, amitriptyline, lidocaine, caffeine) on a 3.0 x 25.0 cm 5m 2-EP column. The gradient elution (5-50% 50:50 MeOH:IPA, 1% DEA, 80 g/min) results in tight chromatographic bands and adequate separation for preparative chromatography. The injection was made into the cosolvent stream so that the sample was introduced to the column head slowly at the initial gradient condition of low solvent strength. The violet trace represents an injection of 500 mg of the mixture (equal masses each compound) on column.

Figure 3. Preparative Scale Separation, Test Standard



CONCLUSIONS

We have investigated the performance of several stationary phases and solvent systems and conclude that generic normal phase chromatographic methods may be developed using an approach similar to the development path of generic RP-HPLC gradient chromatography. The approach may be adapted successfully to larger scales.

Generic gradient RP-HPLC became a valuable methodology when several technological milestones were achieved: LC-MS coupling, stable stationary phases, and scalable chromatography systems. These developments in SFC are in earlier stages of development, but viable and rugged products are in sight.

SELECTED REFERENCES

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