ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN SUPERCRITICAL FLUID CHROMATOGRAPHY

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Introduction

Supercritical-fluid chromatography (SFC) is defined as a mode of chromatography in which both the temperature and the pressure in the column exceed the critical values of the mobile phase. This definition is exact, but rather arbitrary, as there is no phase transition between gases (or liquids) and supercritical fluids. Technically, a gas chromatograph operated above 2.24 atm with He as the carrier gas, is an SFC instrument according to this definition. We normally speak of gas chromatography when retention is largely controlled by the oven temperature (and largely determined by analyte volatility). We speak of SFC when retention is largely controlled by the mobile-phase density (and largely determined by analyte interaction with the mobile phase). Supercritical-fluid chromatography (another name for it is dense-gas chromatography) was first developed in the 1960s by Klesper in Aachen, shortly followed by Sie and Rijnders in Amsterdam. The technique subsided into oblivion during the rapid advent of modern high pressure liquid chromatography (HPLC) in the 1970s. It experienced a second youth in the 1980s. During this period, some researchers optimistically claimed that SFC combined the advantages of gas chromatography (GC) and HPLC. Although statements of this kind still appear in the literature today, the chromatographic community as a whole has come to accept that SFC holds a position somewhere in between, rather than above GC and LC. SFC offers an – occasionally favourable – compromise between the two mainstream chromatographic techniques (see **Table 1**).

Why opt for SFC?

Although this article deals specifically with SFC, we are treating it as a niche technique. In real life, GC and HPLC are more commonly available. When GC can readily be used, SFC offers few advantages other than a lower operating temperature. When

Table 1 General considerations when considering SFC as a possible chromatographic separation method*

Parameter	*GC*	*SFC*	*LC*
(Most) suitable application range	Gases and volatile materials	Low to marginally volatile materials	Low-volatile and non-volatile materials
	All but the most polar analytes	Low to moderately polar analytes	All polarities (non-polar to ionic)
Operating temperature	High (related to analyte boiling point)	Low to moderate	Low
Suitable columns	Packed columns (10–50 μm particle diameters) *Open-tubular columns (100–500 μm internal diameter)*	*Packed columns (3–10 μm particle diameters)* Open-tubular columns (10–50 μm internal diameter)	*Packed columns (1–5 μm particle diameters)* Open-tubular columns (1–5 μm internal diameter)
Suitable detectors	*Vacuum detectors (MS)* *Gas-phase detectors (FID, NPD, ECD, etc.)*†	*Vacuum detectors (MS)* *Gas-phase detectors (FID, NPD*, ECD, etc.)† *Liquid-phase detectors (UV, fluorescence)*	*Vacuum detectors (MS)* *Liquid-phase detectors (UV, fluorescence, refractive index, etc.)*

*The most suitable technique is given in italics.
**Monolithic columns are an emerging alternative to packed columns.
***MS = mass spectrometry.
†FID = flame-ionization detector; NPD = nitrogen–phosphorus or thermionic detector; ECD = electron-capture detector.

Table 2 Possible mobile phases for SFC and their compatibility with different detection principles

Mobile phase	*Polarity*	T_c (°C)	p_c (atm)	*Detection compatibility*			
				FID	*UV*	*MS*	*IR*
Carbon dioxide*	Low	31.05	72.9	++	++	+	±
with modifier:							
Methanol	High	239.4	79.9	−	++	+	−
Formic acid	High			+	+	−	−
Nitrous oxide	Low	36.4	71.5	±	+	+	±
Sulfur hexafluoride	Low	45.5	37.1	±**	+	±	±
n-Butane	Low	152.0	37.5	−	++	±	−
Xenon	Very low	16.6	57.6	+	++	+	++
Ammonia***	High	132.4	111.3	+	+	+	−
Water***	Very high	374.1	217.6	+	+	+	−

*Most suitable mobile phase for most applications.
**Feasible, but highly corrosive.
***Highly corrosive and hardly feasible.

HPLC may readily be used, SFC – when applicable – may offer shorter analysis times and a greater choice of detectors. HPLC can be applied to a much greater variety of samples and analytes than SFC.

Table 1 lists some general considerations for considering or discarding SFC as a possible (analytical) separation technique. The most important reasons for selecting SFC are described below in more detail.

Universal detection When using carbon dioxide (CO_2) as the mobile phase, SFC allows the use of flame-based detectors (see **Table 2**). The flame-ionization detector can be applied almost universally. Even more importantly, it shows an approximately equal response within a class of analytes. As a result, reference standards within each class (rather than for each individual compound) suffice for calibrating a quantitative method.

Because universal detectors are available in GC, but not in LC, there are potentially, two directions in which relevant SFC–FID methods can be developed:

- analysis of non-volatile materials, that cannot be analysed by GC (including the high-temperature version, HT-GC); and
- achieving separations with a (type of) selectivity that cannot be achieved in GC.

Applications in the former direction are quite rare. Some thermally labile components, such as explosives and peroxides, have been analysed by SFC. However, due to the highly inert nature of GC mobile phases (e.g. helium), the increased inertness of modern GC columns, and the increased flexibility of injection systems (e.g. cold on-column injection), such components can often be analysed with good integrity by GC.

Some components that are not sufficiently volatile for analysis by HT-GC may be amenable to analysis by SFC. However, in the author's experience this is limited to highly apolar materials, such as saturated hydrocarbons. For moderately polar analytes, such as aromatic hydrocarbons, HT-GC appears to allow materials with higher boiling points (lower volatility) to be eluted in comparison with SFC.

The most successful SFC–FID methods follow the second approach, using a unique kind of selectivity. In GC, retention is determined by two factors, viz. the pure-analyte vapour pressure and the interactions of the analyte with the stationary phase. In SFC the effect of the vapour pressure can be minimized by working with high-density mobile phases, while the interaction with the stationary phase can be maximized by using stationary phases with large, active surface areas. This allows a so-called 'group-type selectivity' to be achieved, in which the sample is separated (or classified) into a limited number of distinct groups (or classes) of analytes. Within a class, the size (and thus volatility) of the analyte molecules varies, but the chemical structure (functional groups) remains similar. Examples of such methods include the following.

- Separation of complex hydrocarbon mixtures, for example the separation of middle-distillate fuels (diesel or kerosene) into saturates, mono-aromatics and di-aromatics; the determination of the total amount of olefins in gasoline-type fuels. Both these examples concern highly successful applications of SFC. A group-type separation method for middle distillates is standardized as ASTM D-5186. An ASTM standard method for olefins in

gasoline by SFC is expected to be approved by June 2000.
- Separation of (low-molecular-mass) polymers into fractions representing different end-groups.

In both cases, we try to achieve retention that is affected by the chemical structure of the molecules (the *functionality*) but irrespective of their size (molecular mass). This type of chromatography is – somewhat confusingly – referred to as 'critical chromatography', or as 'supercritical-fluid chromatography at the critical conditions'.

Difficult separations SFC possesses some favourable fundamental characteristics, especially in comparison with liquid chromatography. The molecular diffusion is about an order of magnitude greater than in liquids (but three orders worse than in gases) and the viscosity is about a factor hundred lower than that of a typical liquid. Thus, SFC has advantages in terms of mass transfer and column pressure drop. This may result in higher efficiencies per unit length of column, while longer columns may sometimes be used. Therefore, SFC may be attractive for some difficult separations.

SFC has proved a rather attractive alternative to normal-phase LC for the separation of stereoisomers. Like in normal phase LC, CO_2-based SFC features a polar stationary phase and a non-polar mobile phase. Organic modifiers may be added to modify the mobile-phase polarity and detergent-like molecules have been added to help create adequate selectivities. The advantages of SFC in this context are summarized in **Table 3**.

SFC is seen to score well in all categories, except its flexibility in dealing with a variety of samples. Reverse-phase LC (RPLC) also scores well in the table. SFC appears to be more attractive as an alternative to normal-phase LC (NPLC) than to RPLC. The latter technique is compatible with almost all sample solvents, ranging from water to quite non-polar organic solvents, such as tetrahydrofuran. NPLC on unmodified silica surfaces can be used in combination with solvents of low-to-medium polarity. When using polar-bonded phases, again a great variety of sample solvents may be introduced on the column. In both cases (RPLC and NPLC), strongly acidic and strongly basic samples cause problems. SFC is typically restricted to solvents and analytes of low to moderate polarity, especially in case FID detection is to be used.

Preparative separations Carbon dioxide is an outstanding solvent for preparative chromatography. It is available in high purities at a relatively low cost and it can easily be removed from the effluent by evaporation. In fact, the latter characteristic implies that it is somewhat more difficult to collect fractions than is the case in preparative LC.

The main disadvantage of CO_2 for preparative chromatography is its low polarity, which seriously limits its applicability as a chromatographic eluent. Packed columns, with large surface areas and thus high sample capacities, are desirable for preparative separations. Without organic modifiers, only components of little or no polarity can be eluted from such columns using CO_2. When substantial amounts of modifiers need to be used, the advantages of using CO_2 diminish.

Hyphenated systems

SFC–MS Although it would seem that the use of CO_2 is also advantageous when coupling a dense-phase chromatograph to a mass spectrometer (MS), successful SFC–MS systems have hardly materialized. In what are now the most common LC–MS interfaces (electrospray, ESI; and atmospheric-pressure chemical ionization, APCI), a high mobile-phase polarity is preferable. Only a small niche remains where

Table 3 General advantages of (packed-column) SFC in comparison with reversed-phase (RPLC) and normal-phase (NPLC) liquid chromatography*

Property	*Related to:*	*RPLC*	*SFC*	*NPLC*
Efficiency per unit time	Mass transfer (D_m, η)	Second	First	Last
Maximum efficiency	Eluent viscosity (η)	Second	First**	Second
Sample capacity	*Surface homogeneity* Eluent strength	First	Second	Last
Equilibration time	*Surface activity* Mass transfer	First	Second	Last
Flexibility (range of samples)	*Mobile phase* Surface activity	First	Last	Second

*Most important effects are in italics.
**Very high plate numbers have been reached in SFC but operating conditions close to the critical point must be avoided.

SFC–MS may compete with LC–MS, i.e. components of low volatility and low polarity. This implies that there is little incentive for the further development of SFC–MS.

SFC–NMR CO_2 is a perfect eluent when ^{1}H-NMR is to be coupled with a chromatographic separation device. LC–NMR has received a good deal of attention in recent years and some workers have extended this work to include SFC–NMR. The main instrumental difference is that a high-pressure flow-cell (or 'probe') is required. However, the inherent sensitivity of NMR is so low that fractionation followed by offline spectroscopy is usually the preferred approach.

SFE–SFC Very elegant hyphenated systems may arise from a combination of two separation techniques that both involve supercritical fluids. Such systems include SFC–SFC and SFE–SFC. The latter approach, where the extraction serves as an online sample-preparation technique, has been especially investigated by several groups. Unfortunately, the high expectations surrounding SFE around 1990 have not quite materialized. Current interest in SFE–SFC has waned.

Types of SFC Columns

There are traditionally two approaches to SFC. One involves packed columns, the other open-tubular (capillary) columns. This situation is not different from that experienced in GC and LC. In the former technique, open columns are strongly preferred. In the latter, open columns are ideal in theory, but virtually impossible to use in practice. The optimum internal diameter of open columns used in chromatography is essentially determined by the diffusion coefficients of the analytes in the mobile phase. As a rule of thumb, the required analysis time is given by

$$t_R = \frac{N_{req} h d^2}{v D_m}(1 + k)$$

where t_R is the required analysis time for a separation with N_{req} theoretical plates and a solute retention factor of k, h is the reduced plate height, d the column diameter, v the reduced (average) velocity and D_m is the diffusion coefficient of the analyte(s) in the mobile phase. Both N_{req} and k are essentially determined by the retention of the analytes. The greater the selectivity (differences in retention), the lower the required number of plates. Neither N_{req} nor k are affected by the dimensions (length and diameter) of the column. The reduced plate height (h) and the reduced (average) linear velocity (v) have typical values for packed and open-tubular columns. Typically, for packed columns $h = 3$ and $v = 10$, so that $h/v = 0.3$. For open-tubular columns $h = 4.5$ and $v = 45$ are good values, so that $h/v = 0.1$. All things being equal, open-tubular columns are expected to be about three times faster than packed columns.

D_m is the parameter that suggests SFC may allow faster separations than HPLC. However, the diffusion coefficient must be balanced against the characteristic dimension (d) of the column. For packed columns, d is the particle diameter (d_p), while for capillary columns it is the internal diameter of the column (d_c). It follows from the equation that similar performance (in terms of analysis times) can be expected in different forms of open-tubular chromatography when the ratio d_c^2/D_m is kept constant. With $D_{m,gas} \approx 1000 \times D_{m,SF} \approx 10^4 \times D_{m,liquid}$, we find for the optimum diameters of open tubular columns $d_{c,GC} \approx 30 \times d_{c,SFC} \approx 100 \times d_{c,LC}$. Since GC columns have internal diameters between 100 and 500 μm, we anticipate optimal internal diameters for SFC columns to be of the order of 10 μm and for LC columns to be 1–5 μm. Because very many practical problems are associated with the use of such extremely small columns, open-tubular SFC (OT-SFC) has typically been performed with somewhat larger columns (50 or 100 μm). However, this has led to a modest efficiency and speed.

Table 4 provides a summary of some of the advantages and disadvantages of using packed and capillary columns in GC and LC, with a more extensive summary of the characteristics of packed and open-tubular SFC. In SFC, open-tubular columns with optimal diameters are difficult to use. As a result, packed-column SFC is the more robust and more practical technique.

Most Important Parameters

The parameters that are most important in the development of SFC methods are as follows.

Mobile-Phase Density

The outstanding parameter in SFC is the mobile-phase density. This factor plays a role similar to the temperature in GC and the solvent strength in LC. Density gradients (typically increasing density linearly with time) in SFC are the common equivalent of temperature gradients in GC and mobile-phase composition gradients in LC. When the density increases,

Table 4 Advantages (⇑) and disadvantages (⇓) of packed and open-tubular (capillary) columns in GC, SFC and LC

	GC	*SFC*	*LC*
Packed columns	⇑ Fast analysis	⇑ Compatible with back-pressure regulators	⇑ Compatible with many detectors
	⇑ Large sample capacity	⇑ Broad range of optimum *k* values	⇑ Fast analysis
	⇑ Broad dynamic range	⇑ Programming often not needed	⇑ Large sample capacity
	⇑ Reliable and robust	⇑ Fast analysis	⇑ Broad dynamic range
	⇑ Allows preparative separations	⇑ Large sample capacity	⇑ Reliable and robust
	⇔ Perceived to be old-fashioned	⇑ Broad dynamic range	⇑ Allows preparative separations
	⇓ Low permeability	⇑ Reliable and robust	⇔ Columns still not perfect
	⇓ Limited maximum efficiency	⇑ Easy online solvent mixing	⇓ Very small particles required
		⇑ Routine loop injections	⇓ Low permeability
		⇑ Allows preparative separations	⇓ Limited maximum efficiency
		⇔ Columns optimized for LC	⇓ High pressure drop
		⇓ Low permeability	
		⇓ Limited maximum efficiency	
		⇓ High pressure drop	
		⇓ Active stationary-phase surface	
		⇓ Modifiers often required	
		⇓ FID often not possible	
Open-tubular columns	⇑ High permeability	⇑ Inert surface for polar analytes	⇓ High permeability
	⇑ High maximum efficiency	⇑ Modifiers not often needed	⇑ High maximum efficiency
	⇑ Inert surface for polar analytes	⇑ FID can usually be used	⇔ Extremely low flow rates
	⇔ Many different injectors	⇑ High permeability	⇓ Very few detection options
	⇓ Limited sample capacity	⇑ Low column pressure drop	⇓ Extremely small diameters required
	⇓ Limited dynamic range	⇑ High theoretical efficiency*	⇓ Extremely small sample volumes
	⇓ Sensitive to (large volumes of) solvents	⇔ Very low mobile-phase flow rates	⇓ High detection limits
	⇓ Sensitive to (liquid) water	⇓ Very small diameters required	⇓ Extremely small dynamic range
		⇓ Sub-optimal (too large) columns commonly used	⇓ No tolerance for extra-column dispersion
		⇓ Little tolerance for extra-column dispersion	
		⇓ Very small sample volumes	
		⇓ Proper injections are difficult (very small volumes and time splitting)	
		⇓ Rather high detection limits	
		⇓ Very small dynamic range	
		⇓ Hard to combine with MS	
		⇓ Narrow range of optimum *k* values	
		⇓ Sensitive to (large volumes of solvents	
		⇓ Programming usually required	
		⇓ Requires fixed restrictor (no adequate flow control)	

*In practice, the efficiencies obtained in open-tubular SFC are well below the theoretically expected values.

interactions between the mobile phase and the analytes increase. The analytes are better dissolved in the mobile phase. Retention typically decreases exponentially with increasing density.

The column inlet and outlet pressures are significant parameters, but their effect on retention is indirect, as the pressure affects the density. In this context, the pressure closest to the critical value is most important. In SFC this is the column-outlet pressure.

Pressure, temperature and density are connected through an equation of state. Different equations can be used that provide good estimates for the density of pure supercritical fluids. However, when mixed eluents are used (e.g. CO_2 with a modifier such as methanol), no reliable equation is available that provides the density as a function of pressure and temperature. Nevertheless, when the latter two parameters and the composition of the eluent are established, the density is in principle defined.

Temperature

The second most important parameter is the temperature. Like the pressure, the temperature has a significant effect through its effect on the density. However, at constant density, an increased temperature may lead to a lower retention, especially for relatively volatile analytes. Apart from the temperature of the column oven, the temperature of the injector or injection valve can also be quite significant. In order to test the feasibility of eluting certain analytes by SFC, it is worthwhile to perform some experiments at an increased injector temperature.

Stationary Phase

The stationary phase. The column used plays a major role in SFC, especially when using non-polar mobile phases, such as carbon dioxide. The stationary phase has a large effect on the retention and an often prevailing effect on the selectivity. In addition, the stationary phase has a very large effect on peak shape and peak width (efficiency). Again, this effect is strongest when using non-polar eluents (CO_2).

Studying the effects of different stationary phases can be (very) expensive and time consuming. There are often practical limitations and when the option to use modifiers is available, this may be attempted first, provided UV detection is adequate.

Mobile-Phase Composition

The mobile-phase composition may have dramatic effects on retention, selectivity, efficiency and peak shape in SFC. However, adding modifiers has some significant disadvantages, especially with regard to detector compatibility. Therefore, changing the mobile-phase composition is not the first option in developing an SFC method. There are two modifiers that allow FID detection to be used, i.e. water and formic acid. Both have been investigated, but neither has found many applications in practice. The effect of a modifier tends to be greatest at low concentrations. In this range, the modifier mainly acts by competing with the analytes for strong adsorption sites on the stationary-phase surface. A small amount of modifier (often well below 1%) may lead to a much reduced analysis time and a much increased column efficiency. The use of modifiers often leads to much sharper and much more symmetrical peaks. At higher concentrations, modifiers may still affect retention and selectivity, through increasing the polarity and the density of the mobile phase. However, these effects are much smaller and the variations become more gradual. At these high concentrations it is more likely that different modifiers give rise to substantially different selectivities.

Method Development

The flow chart for developing an SFC method shown as **Figure 1** follows logically from the discussion on different types of columns and the overview of main parameters given in previous sections above.

Carbon dioxide will almost always be the eluent of choice. This is assumed to be the case in Figures 1 and 2. Instrument availability is the obvious first consideration. It greatly affects all the other decisions taken. Selecting packed columns is very attractive (see Table 4), but this requires a compatible instrument, with a pumping system capable of delivering substantial flow rates. It is quite possible to use microbore (1-mm i.d.) and packed-capillary (≤ 0.5 mm i.d.) in SFC, but some of the advantages of packed-column SFC are then lost. Most importantly, miniaturized columns do not allow the use of controllable back-pressure regulators. In this case, there is no adequate flow control, a problem that is especially serious when the mobile-phase density (in practice the pressure and/or the temperature) is programmed during the run.

When a novel sample is being subjected to SFC, the recommended strategy is to rapidly establish

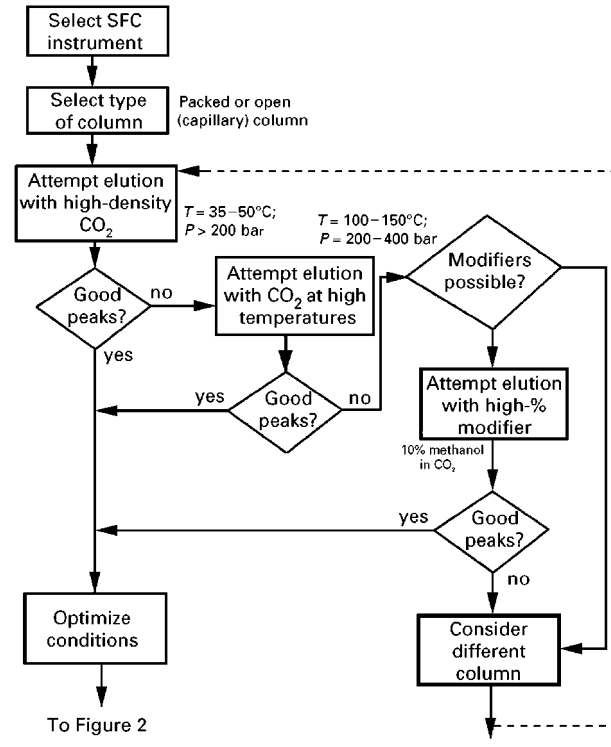

Figure 1 Flow chart for the development of an SFC method.

whether the analytes can be eluted. Because retention decreases with increasing density, high densities must be tried first. Once sharp peaks have been obtained for the analytes it will be easy to increase the retention by lowering the density. In open-tubular SFC a mobile-phase density gradient with a high final density will typically be used. In packed-column SFC, where retention times are typically of the order of minutes, constant elution conditions (isobaric, isothermal, isochoric and isocratic) will be preferred for initial scanning experiments.

Despite the high praise for FID, it is extremely valuable to have an informative detector available at this stage. A UV detector, especially a multichannel diode-array (DAD) instrument, will provide much on-line information on the progress of the method development. It is very much easier to know the whereabouts of different analyte peaks in the chromatogram if DAD and FID data are obtained simultaneously. In open-tubular SFC, a DAD cannot be used and SFC–MS is the obvious choice. However, SFC–MS is not an easily accessible practical tool.

If the analytes cannot be eluted at the highest possible (or highest practical) CO_2 density, it may yet be worthwhile to attempt elution at elevated temperatures. Increasing the temperature may lead to lower densities, but this may be compensated by an increased analyte volatility, especially for analytes with a significant vapour pressure. In addition, adsorption effects (including analyte-stationary-phase interactions) may be reduced. The temperature of the injector plays a different role. Sometimes it has proven beneficial to inject at temperatures well above the column temperature. In many cases, the sample (or sample solvent) is a limiting factor. When loop injection is used, the temperature must usually be kept well below the boiling point of the solvent.

If the analytes are not eluted as sharp, symmetrical peaks at high densities, nor at increased temperatures, then the use of modifiers may be attempted if this is an option. Using pre-mixed CO_2-based mobile phases is not attractive for reasons of accuracy and reproducibility as the composition in the cylinder will vary with time. Also, the flexibility regarding the possible concentrations is very limited. However, this is often the only choice in miniaturized (open-tubular) systems. In some cases, mixtures have been prepared inside the pump head of a syringe pump, which is preferred in terms of accuracy and flexibility. Many packed-column SFC systems allow more convenient online mixing, which makes it relatively easy to investigate the possible advantages of using modifiers. Unless experiments are performed with water or formic acid as a modifier (neither being very practical), the use of FID is not feasible at this point. It may be useful to attempt a few different modifiers. However, the chances of obtaining good peaks become very small once the addition of 10% methanol has proven inadequate for the purpose.

The scanning experiments suggested so far can typically be performed within one or two days. This is what is referred to when it is claimed that method development in SFC can be very rapid. If at this stage the results are unsatisfactory, an important decision needs to be made. It is quite possible that better results will be obtained on a different column. However, when it is decided to investigate the use of different columns the amount of work needed will be multiplied. If there are still good reasons to opt for SFC, then it is most realistic to identify the column with the most inert surface (for example, a column packed with polysiloxane-coated particles) and repeat the sequence outlined above. If this attempt is not successful, then an alternative separation technique must be seriously considered.

Method Improvement and Troubleshooting

If at any stage during the method development rapidly eluting, sharp peaks have been obtained for the analytes, then the separation can be optimized. The actions that may be taken are summarized in **Figure 2**.

From the initial results it may be concluded whether the retention should be increased. The appropriate action depends on the stage at which success was obtained. If high-density CO_2 at a low temperature proved successful, then reducing the density will suffice. When elevated temperatures were

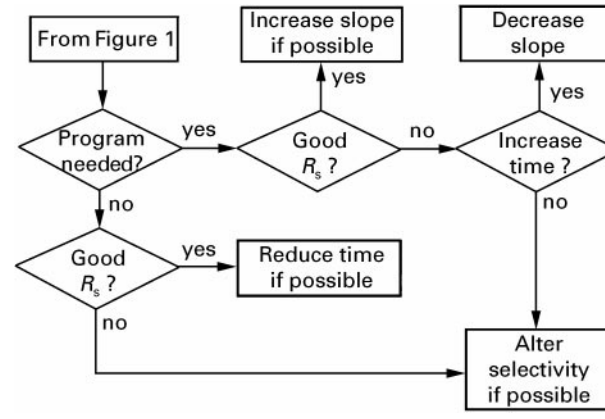

Figure 2 Flow chart for the optimization of an SFC method. R_s denotes resolution (i.e. ratio of distance between two peaks and their average base width).

used, the temperature may be lowered and/or the pressure may be decreased to achieve optimal elution conditions. If a modifier was used then the concentration of modifier may be lowered or the mobile-phase density may be decreased.

When moving the retention of the analytes into the optimum range, it will become apparent whether or not programmed elution is needed. In packed-column SFC this will only be the case if the last analyte has a high retention factor (say $k > 15$) when the first eluting analyte has $k = 1$. In marginal cases, it may be possible to use a different (less selective) stationary phase to avoid programmed analysis. In open-tubular SFC, programmed analysis is often needed just to deal with the excess of solvent introduced with the analytes. Resolution in programmed analysis can typically be increased by lowering the eluent strength (in SFC typically the density) at the start of the program and by lowering the slope (increasing the duration of the gradient segment of the program). In either case, this leads to a longer analysis time. In open-tubular SFC (or when using a fixed restrictor in packed-column SFC) the flow rate may be relatively high, especially in later parts of the program. In this case lowering the flow rate (by preparing a smaller restrictor) may be more rewarding, either by itself, or in combination with lowering the gradient slope.

If a separation under non-programmed conditions leads to abundant resolution between the relevant analytes, it may be possible to decrease the retention time. In order of decreasing rewards, this may be achieved by decreasing the column length, increasing the flow rate, or increasing the eluent strength (increasing the density or the modifier concentration). In the more important case in which the achieved resolution is inadequate, the pressure and/or temperature may be altered, but this often affects retention much more than selectivity (i.e. the retention factors of the various analytes tend to be affected in the same way). If modifiers are being used, different modifiers may lead to different selectivities. However, the most likely road to success is to attempt different stationary phases at this stage.

When we considered the use of different stationary phases at the end of the method-development stage, this was thought not to be very promising. However, in the present situation, at the method-optimization stage, it has already been demonstrated that SFC is a feasible technique for eluting the analytes, but not yet for separating them. Trying different stationary phases with greatly different selectivities may be a rewarding option at this stage.

The actions outlined here may also be relevant when the separation deteriorates at some stage during the development or application of an SFC method. When this is the case, proper functioning of the equipment should first be verified. The mobile-phase density (pressure and temperature), flow rate and composition may all be verified. If variation in either of these parameters is excluded, then a change in the stationary-phase surface is a probable diagnosis. The column may be simply replaced at this stage, but a few other options are open. These are listed below.

- It is possible that the column is contaminated with very 'heavy' (high molecular weight) or very polar material from the sample or the solvent, that cannot be eluted under SFC conditions. In this case it may be possible to wash the column with a liquid solvent such as 2-propanol, to recondition it in the SFC instrument (without the FID connected), and to use it again for the application.
- It is possible that the column is 'irreversibly' altered by the presence of sample or solvent components. Water on a silica column is the most obvious example. Water may be removed by drying a column overnight at a high temperature (e.g. 250°C) under a small flow of an inert gas (N_2, H_2 or He). A GC oven is very useful for this purpose.
- In case non-programmed conditions are used, it may be advantageous to program the column to different conditions at the end of each analysis, each series of samples, or each working day to avoid column contamination.
- Some columns may change gradually in a truly irreversible manner. The use of amino-derivatized columns is not recommended in combination with CO_2, due to the anticipated formation of carbamates. If such a column is to be used, a gradual change of the stationary phase may necessitate gradual adaptation of the mobile-phase density or composition to maintain adequate resolution. Less dramatic changes of the surface may occur with different stationary phases (e.g. a gradual loss of some chemically bonded ligands from the surface) and these may also be counteracted by small changes in the conditions, rather than by frequently replacing the column.

See also: **II/Chromatography: Supercritical Fluid:** Historical Development; Instrumentation; Large-Scale Supercritical Fluid Chromatography; Theory of Supercritical Fluid Chromatography.

Further Reading

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ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN THIN-LAYER (PLANAR) CHROMATOGRAPHY

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Introduction

One of the most critical steps of qualitative and quantitative planar (thin-layer) chromatographic (TLC) analysis is development of a method resulting in sufficient separation. The main steps of method development are summarized in **Figure 1**. The first stage is selection of the stationary phase, the vapour phase, and suitable solvents. This stage is the *sine qua non* of method development, and the selection of these can occasionally immediately result in a suitable separation. For most real separation problems the second stage, optimization of the mobile phase is also necessary. The third part of method development is selection of the final conditions, for example the mode of development, transfer of the mobile phase to an appropriate forced-flow method, and last but not least, the selection of suitable operating parameters. This paper gives essential guides to method development in planar chromatography and draws attention to the most important considerations.

Stationary Phase Selection

TLC separations can be performed on modified, unmodified, and impregnated stationary phases, because of differences between the chemical properties of the sorbent material and those of compounds present in the sample to be separated. Different types of chromatographic process (normal-phase, reversed-phase, partition, and ion exchange chromatography) can be distinguished on the basis of the types of interactions involved. Although more than 90% of TLC separations are performed on silica, chemically bonded phases have recently become increasingly popular for solving special separation problems.

In normal-phase chromatography the hydroxyl groups on the surface of the silica are the polar, active centres which result in the interactions leading to the retention of the compounds to be separated. These interactions are mainly hydrogen-bonding and induced dipole–dipole interactions. The stationary phase can generally be characterized in terms of its specific surface area, specific pore volume, and mean pore diameter.

Unmodified stationary phases include silicas, aluminas, kieselguhr, silicates, controlled-porosity glass, cellulose, starch, gypsum, polyamides, and