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**Highlights**

The novel SFC feed injector that adds the sample to the mobile phase stream was investigated. Mixing of sample with mobile phase increases sample volume and dilutes sample solvent. Optimal feed speed is a trade-off between injection solvent mismatch and sample dilution. The overfeed volumes increases band broadening and is best replaced by an apolar solvent. Partial sample injection can further decrease injection band broadening.
Effect of the feed injection method on band broadening in analytical Supercritical Fluid Chromatography

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Abstract

The behavior of a novel type of SFC injector, the feed injector, was investigated. In SFC, the sample compounds are usually diluted in a solvent which has a higher elution strength than the mobile phase, which leads to solvent mismatch upon injection and evidently band broadening. The feed injector differs from standard injectors as the sample, contained in the sample needle or loop, is not switched in line with the mobile phase flow, but directly injected/added to the mobile phase flow (F). The subsequent mixing of sample and mobile phase flows inherently results in a dilution of the sample, thus reducing the solvent mismatch. However, for a given injection/feed flow rate $F_{\text{feed}}$, the total volume in which the sample is contained increases with a factor $(F_{\text{feed}}+F)/F_{\text{feed}}$. In addition, to ensure that all of the loaded sample is injected on the column, an additional overfeed volume ($V_{\text{ov}}$) needs to be injected after the sample plug. To better understand the effect of these operating parameters, a wide range of injection conditions was investigated by varying the $F_{\text{feed}}/F$-ratio, $V_{\text{ov}}$, overfeed solvent etc. under SFC conditions. It was found that an optimal $F_{\text{feed}}/F$ exists which is independent of $F$ and decreases with increasing solvent strength dependency of the sample compound. Decreasing $V_{\text{ov}}$ has a beneficial effect on peak dispersion but can only be varied over a certain range to ensure the full injection of the loaded sample. On the other hand, it was found that a much larger gain could be made by switching the overfeed solvent to one more compatible with the CO2-based mobile phase. Further reduction of the band broadening could be achieved by applying partial sample injections.

Keywords

supercritical fluid chromatography; feed injector; band broadening; feed flow rate; overfeed volume; sample solvent
1. Introduction

The goal of any automated sample injector is to introduce a precise volume of sample solution from a sample vial at atmospheric pressure into the mobile phase stream under high-pressure conditions. It should be capable of high precision and long-term reliability [1]. The sample introduction in any chromatographic system inherently contributes to the extra-column band broadening due to the size of the sample plug, the method of sample drawing and injection procedure and the flow path between sample plug and injector outlet capillary. When the sample is dissolved in a solvent that deviates from the initial mobile phase composition of the chromatographic method, additional dispersion can occur when the sample plug reaches the head of the column. For Supercritical Fluid Chromatography (SFC), the need to keep the entire flow path pressurized to avoid decompression of the mobile phase (mainly CO₂), puts additional limitations on the injection method, as the entire sample loop volume needs to be decompressed before sample loading. It inherently also results in a difference between the sample solvent (usually an organic solvent or a mixture thereof) and the mobile phase (CO₂ + co-solvent), as it is impractical to prepare and store the sample in the high pressure mobile phase solvent. The flow-through needle (FTN) design, i.e., the most common method in LC, is not suitable for SFC systems as the injection flow path passes through the sample loop, needle, seat and seat capillary, which are exposed to atmospheric pressure during sample load. The (de)compression of such a large volume would cause several practical problems such as the evaporation of the mobile phase during decompression and a large pressure drop upon injection when the loop is recompressed. Therefore, the standard injection method in analytical SFC is the loop type injection, where a small volume sample loop (1 - 20 µL) is (partially) filled with the sample. For preparative scale SFC, the so-called modifier stream injection is also often used [2,3,4]. Although the injection principle is the same, here the sample loop is not placed after the mixing of the CO₂ stream and modifier as is the case in analytical SFC instrumentation, but the injection is made in the modifier-stream, before mixing with the CO₂ stream. Although beneficial for the very large volume-injections in prep-SFC, this technique is not used for analytical SFC separations.

An alternative type of injector, combining the flexibility in sample volumes of the FTN with the high-pressure injection requirements of SFC, is the so-called feed injection that was recently introduced in a commercial SFC instrument by Agilent Technologies (1260 Infinity II SFC System). In this injector, the very flexible and high precision injection volumes of the flow-through needle type injectors are combined with the requirement to maintain the operating pressure in the sample flow path as for the fixed loop injector (see Fig. 1). The system resembles an HPLC flow-through needle injector as the required sample volume is drawn into the sample needle at atmospheric pressure, after which the sample needle is moved back to the needle seat and sealed for high pressure operation. This is followed by a compression of the sample path to the operating pressure of the system (i.e. pump pressure). In a next step, the injector valve that previously only allowed flow from the pump to the column is switched so that both the flow path from the pump and sampling path needle are connected to the
mobile phase flow towards the column. The injector pump pushes, or ‘feeds’ the sample volume, together with a certain overfeed volume of additional solvent present in the needle/sample loop taken from a dedicated solvent bottle, into the mobile phase flow at a set flow rate (max. 1 ml/min). This additionally injected overfeed volume \( V_{OV} \) is to ensure that the full sample volume accurately metered during sample drawing is injected in the system. Fig. 1 provides a basic schematic representation of the injection method. Fig. 1a represents the end of the load step during which the sample is drawn into the needle, returned to the needle seat and followed compression of the sample loop. Fig. 1b represents the valve position during injection, when the sample and the overfeed are joined with the mobile phase stream. A detailed drawing of the entire injection module (including e.g. wash steps), can be found in the manual of the instrument [5].

In SFC, regardless of the type of injector, the sample solvent (usually an organic solvent) is always significantly different from the mobile phase (mainly compressed CO\(_2\)). Whereas the mobile phase is rather apolar, the typically used sample solvents that have a high solubility for the sample compounds have a much higher elution strength than the mobile phase, causing a mismatch in retention at the head of the column when the sample is injected [6-10]. In addition, the sample solvent itself can interact with the stationary phase, causing a disturbance in the adsorbed layer of mobile phase modifier (often MeOH in SFC). All these effects can cause significant distortion of the sample band, causing significantly additional band broadening or even strong peak deformation (tailing/fronting) of the peaks. The effect of sample solvent in SFC was investigated in detail by Desfontaine et al. for a wide range of sample compounds, sample solvents and stationary phases [11]. They concluded that the sample solvent effect depends not only on the sample solvent but can also vary significantly for different sample compounds or stationary phases. In a combined experimental and modeling study, Enmark et al. found that (1) solvent strength mismatch is the main reason for peak distortion, (2) the adsorption of MeOH to the stationary phase increases the band broadening and (3) viscous fingering gives additional broadening of the solute bands [2].

The use of a feed injector partially alleviates the effects of solvent mismatch between the mobile phase and the sample solvent, as during the injection the sample is mixed with the mobile phase stream. As a result, the sample is diluted proportional to the ratio of the mobile phase flow rate \( F \) and the feed flow rate \( F_{feed} \). Taking for example a simplified case (ideal plugs and incompressible flow) where the sample solvent is the same as the mobile phase modifier which is set at 10% (volume-based) at the start of the method. Since \( F_{feed} = F \), the sample solvent will be diluted to 55% (v/v) modifier and 45% (v/v) CO\(_2\), rather than pure (100%) solvent. For \( F_{feed} = F/3 \), the sample will already be diluted to 32.5% (v/v) modifier. As a result, a lower \( F_{feed} \) leads to a more “compatible” sample solvent. Following a similar train of thought, also effects of viscosity mismatch between sample plug and mobile phase can be reduced.

The inherent downside of this dilution is of course that also the sample concentration is decreased as the total sample volume is increased proportional to \( F/F_{feed} \) (in fact it increases with a factor \( (F_{feed}+F)/F_{feed} \)). Abrahamsson and Sandahl showed that by increasing the system...
volume before the column, and as such improving the mixing between sample and mobile phase, the effect of the sample solvent on performance became less pronounced, albeit at the cost of an increase extra-column dispersion \[7\].

The dilution of the sample with the mobile phase solvent is somewhat related to the modifier stream injection technique that is used in prep-SFC as mentioned earlier \[2,3,4\]. Since the sample is introduced in the modifier stream, the subsequent mixing with the CO\(_2\) stream also dilutes the sample and decreases the sample solvent concentration. In fact, if the sample solvent and modifier are the same, the sample ends up with the same composition as the mobile phase, thus eliminating any composition mismatch. The downside of this method is that the dilution ratio is fixed by the modifier/CO\(_2\) ratio and thus sometimes the sample plug gets diluted (and the injection volume increased) more than would be required to avoid excessive sample solvent/mobile phase mismatch effects. For the feed injector, this dilution can be chosen freely within the limits imposed by the maximum injection feed flow rate of 1ml/min. In addition, the sample plug needs to pass through the mixing chamber in case of the modifier stream injection, which greatly adds to the dispersion of the sample plug, especially if this would be applied on an analytical scale SFC application.

It is thus of interest to investigate the trade-off between the advantages and disadvantages of the sample solvent dilution vs. the sample volume increase. In addition, the injection of the overfeed solvent results in the introduction of an extra plug of strong solvent in the column after the sample plug. The goal of this experimental study is therefore to investigate how the different feed injection parameters (\(F_{\text{feed}}, V_{\text{ov}}, \) overfeed solvent and injection procedure) affect band broadening in SFC. Other aspects, such as linearity, accuracy and precision, although certainly not irrelevant, are not the focus of this work and can be found in the specifications of the instrument.
2. Experimental

2.1 Chemicals and columns

Methanol, hexane and isopropyl-alcohol (IPA) (LC-MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands), CO$_2$ was purchased from Air Liquide (Paris, France). Testosterone (>99%), progesterone (>99%) and $\beta$-estradiol (>98%) were purchased from Sigma-Aldrich (Overijse, Belgium). The analytes were dissolved at a concentration of 100 $\mu$g/mL in pure MeOH. For the measurements where also very low injection volumes were investigated, a concentration of 500 $\mu$g/ml was used to obtain high enough UV signals. Some experiments were also performed with a 15/15/70 v%/v%/v% MeOH/IPA/hexane mixture as sample solvent, where the IPA was added to the mixture to allow better mixing of MeOH and hexane [12]. Since the improvement in performance with this sample solvent mixture is in agreement with earlier work [12,13] and are thus not specific for the feed injector, these results are not discussed in detail. A Zorbax RX-SIL 4.6 x 150 mm with 5 $\mu$m particles was used for all experiments (Agilent Technologies, Waldbronn, Germany).

2.2 Instrumentation and conditions

The SFC system used was an Agilent 1260 Infinity II SFC system, equipped with the following modules: SFC Control Module (G4301A), SFC Binary Pump (G4767A), SFC Multisampler (G7116A), Multicolumn Thermostat (G7115A) and Diode Array Detector (G7165A). The column thermostat and detector where equipped with a Standard flow Quick connect heat exchanger (V = 1.6 $\mu$L) and a 2 $\mu$L SFC flow cell (3 mm path length) respectively. Data was recorded at a wavelength of 254 nm (progesterone) and 210 nm (testosterone, $\beta$-estradiol) and a frequency of 40 Hz. The oven temperature was always set at 30°C and the back pressure at 150 bar. The mobile phase consisted of 12v% MeOH in CO$_2$ and flow rates were set at 1.25, 2.5 or 3.75 mL/min. These rather ‘mild’ conditions (high modifier fraction and backpressure) were chosen to avoid the region of high mobile phase compressibility around the critical point (and its resulting strong effects on retention and performance), as this could cause secondary effects not related to the feed injector. All reported flow rates are those set in the instrument. Since the SFC Control Module delivers the compressed CO$_2$ just below operating pressure to the SFC Binary Pump, the latter only increases the mobile phase pressure by around 10 bar. Since the flow rate metering occurs in the SFC Binary Pump, which operates at room temperature, these volumetric flow rates are very close to the actual mobile phase flow rates during injection. In addition, since the system pressure drop was only 54bar at 2.5ml/min, no significant variations on the flow rate is expected throughout the system and it was found that the additional complexity of adding mass flow meters in the system was not required.

The injection volume was 1.25, 2.5 or 5 $\mu$L, while the injection flow rate (feed speed) was varied between 100 – 1000 $\mu$L/min. MeOH or hexane were used as overfeed solvent and the overfeed volume ($V_{ov}$) was either fixed at 4 $\mu$L or varied between 0 – 10 $\mu$L. All injections were performed in triplicate and the average value was reported in all figures. With the exception
of the data presented in Fig. 9, in all other cases the + 1σ error bars were smaller than the symbols used in the plots and were therefore omitted. To determine peak variances, the method of moments was used.

For this first detailed investigation of the effect of the different injection method settings of the feed injector, it was chosen to limit the number of experimental variables. Therefore, only three structurally similar compounds, that however vary in retention factor and retention dependency with mobile phase composition, were investigated. To be able better to quantify the effects due to the injection method, the conditions/components were also chosen such that the components were only weakly retained (smaller column dispersion contribution). In addition, only a single stationary phase and one mobile phase modifier (MeOH) without additives were used. In most cases, the sample and overfeed solvent were the same as the mobile phase modifier, to avoid additional interactions between sample solvent and the stationary phase that are often very specific for a given combination of sample/stationary phase [11].

3. Results and Discussion

For the feed injector, two important parameters can be set besides the injection volume $V_{inj}$, i.e., the feed flow rate (0 – 1000 µL/min) and the overfeed volume $V_{ov}$ (0 - 10µL). As mentioned in the introduction, a decrease in injection flow rate causes an increase in peak volume (broader sample plug), but with a more diluted sample solvent, i.e., with a composition closer to the mobile phase.

3.1 Effect of feed flow rate on peak width

In a first set of experiments, a series of injections were performed with varying feed flow rate $F_{feed}$, but with at a fixed mobile phase flow rate $F$. The experiments were performed at three different fixed $F$ (1.25 mL/min, 2.5 mL/min and 3.75 mL/min) and three different $V_{inj}$ (1.25 µL, 2.5 µL and 5 µL), with a fixed $V_{ov} = 4µL$.

Figure 2a shows example chromatograms at $F=2.5$ml/min for a selection of injections performed with the different $F_{feed}$-values that were investigated. The chromatograms clearly show that the peak widths and heights vary with $F_{feed}$, but also that the highest and narrowest peaks are not obtained for the highest $F_{feed}$ of 1000 µL/min, which corresponds to the least dilution/smallest volume of the peak. To look at this in more detail, Figs. 2b-d show a zoom-in on each of the three different peaks. For the first eluting compound (progesterone), a feed flow rate of 500 µL/min (red) seems to yield the highest and narrowest peak, whereas for the second (testosterone) equally high and narrow peaks are observed for 250 and 500 µL/min. The chromatograms of the last eluting compound clearly show that 250 µL/min yields the highest and narrowest peak. In addition, a clear trend in the location of the peak apices is observed: the lower $F_{feed}$ the later the apex. This is not surprising, as for stronger dilution of
the sample plug (lower \(F_{\text{feed}}\)), the sample enters the column in a weaker solvent and is thus initially more strongly retained, yielding higher retention times. The lower feed flow rate also causes a small delay of the timing with which the mean of the sample plug enters the column due to increased plug length. The observed delays were however larger than can be expected from this effect alone.

In order to more quantitatively describe the effect of \(F_{\text{feed}}\) on band broadening, the peak variances \(\sigma_t^2\) were calculated and plotted vs. the ratio of \(F_{\text{feed}}/F\), as shown in Fig. 3a for the three investigated sample compounds. The x-axis thus represents an inverse measure for how much the sample plug is diluted, i.e., lower \(F_{\text{feed}}/F\) values correspond to a stronger dilution of the sample plug. It is directly clear that for each compound, an optimal \(F_{\text{feed}}/F\)-ratio exist. This ratio thus corresponds to the point where the best trade-off between the wider sampler plug resulting from the lower \(F_{\text{feed}}\) and the decrease in sample solvent mismatch is obtained. The location of this optimum is however different for the three compounds. In addition, since the column contribution to peak variance increases with (1+k)^2, the more strongly retained compounds obviously have larger \(\sigma_t^2\)-values.

Whereas Fig. 3a shows the result for 3 compounds at a fixed \(F\), Fig. 3b shows the normalized peak variances \((\sigma_t^2/\sigma_{t,\text{min}}^2)\), i.e. normalized with the minimum \(\sigma_{t,\text{min}}^2\) of the fit, see below for fitting details) for a single compound (\(\beta\)-estradiol), but obtained at different flow rates. The normalization was done to eliminate the effect of flow rate on the column contribution to band broadening. Fortunately, exactly the same optimal value of \(F_{\text{feed}}/F\) for the different flow rates was obtained, limiting the required number of experiments. This observation is not surprising since the dilution is proportional to \(F_{\text{feed}}/F\) such that the same increase in peak width and reduction in solvent strength is obtained. Similar conclusions could be drawn for the two other sample compounds (results not shown).

Since only a finite number of different \(F_{\text{feed}}\) values were tested, the curves in Fig. 3 were fitted around their minimum with a 3rd order polynomial equation using MS Excel to be able to calculate the optimal \(F_{\text{feed}}/F\)-ratio. The latter values are given in Table 1 for the different investigated flow rates and injection volumes. As already mentioned, the flow rate has little to no effect on the optimal \(F_{\text{feed}}/F\) ratio. On the other hand, the optimal ratio appears to decrease with decreasing sample volume. It is likely that this is the result of the overfeed volume which was kept constant at 4 \(\mu\)L. As a result, for a smaller volume a relatively much larger plug of strong solvent is injected after the sample, causing additional peak defocusing. A stronger dilution of this strong solvent plug (lower \(F_{\text{feed}}/F\)) counters this. The effect of the overfeed volume and solvent is investigated in more detail in the next section (3.2).

It was already clear from Fig. 3a and can also be seen in Table 1 that the optimal \(F_{\text{feed}}/F\)-ratio depends on the sample compound, where the more retained compounds appear to require a stronger dilution of the sample plug (lower optimal \(F_{\text{feed}}/F\)). To better understand this behavior, the retention factor of the three compounds was measured as a function of the modifier fraction in the mobile phase and fitted according to the linear solvent strength model
Figure 4 shows that the investigated compounds with a higher retention factor also have a much larger solvent strength dependency (S-factor). Previous studies showed that solvent strength mismatch is one of the main reasons for peak distortions [2] and this mismatch decreases for lower values of $F_{\text{feed}}/F$. It thus seems that the S-factor mainly determines the optimal $F_{\text{feed}}/F$-ratio since larger S-value result in a larger relative difference of k in the sample solvent plug when it enters the column and k in the normal mobile phase, for a given difference in composition.

3.2 Effect of overfeed volume and solvent

The second important parameter of the feed injector is the overfeed volume. Increasing the overfeed volume ensures that more of the sample volume drawn into the needle is injected but comes at the cost of a larger plug of strong solvent (methanol) injected after the sample band. In most applications, it is however not essential to inject the full sample volume or know the true injection volume as anyhow a calibration curve (peak area vs. injected concentration) is used for quantification. So, as long as the precision of the injector is high, injecting only part of the entire volume is not a huge problem, as long as the method does not need to be transferred to a different instrument. Fig. 5a shows the effect of overfeed volume on normalized peak area (maximum overfeed volume of 10 µL was taken as the reference). It can be seen that, for an injection volume of 1.25 µL with no overfeed volume, only 55% of the sample is injected while with an overfeed of 3 µL this amounts up to 90%. This is not unexpected, because, due the parabolic flow profile in the open tubes of the needle and sample loop, the maximum velocity (in the center) is twice that of the average velocity. In combination with the time required by the injector to move from the sample load to the injection position, more than the sample volume is required to inject the entire sample (see e.g. Fig. 4 in [15]).

Fig. 5b plots the corresponding normalized peak variance as a function of $V_{\text{ov}}$, using the peak variance at $V_{\text{ov}} = 0$ µL as the normalization factor as this corresponds to the smallest peak width. A strong linear increase in peak width can be observed with increasing overfeed volume, reaching an increase in peak variance by a factor of 4 (i.e., a doubling of the peak width) for the largest $V_{\text{ov}}$ of 10µL. Looking in more detail, a factor of 5 is found for progesterone and a factor around 3.75 for the two other compounds. This is since the early eluting progesterone has the narrowest peak width and thus the smallest $\sigma^2_{t,V_{\text{ov}}=0}$ used for normalization. The absolute increase in $\sigma^2_t$ is the largest for $\beta$-estradiol (see also Fig. 5c), for the same reason as why it has the lowest optimal $F_{\text{feed}}/F$ ratio discussed in the previous paragraph, i.e. because it has the strongest dependency of retention modifier fraction. The combined effect of the increase in injected sample amount (Fig. 5a) and increase in peak width (Fig. 5b) however results in a maximum sensitivity (highest peak height) for an overfeed volume of around 1.25 - 1.5 µL. For an easy comparison (see next paragraph), Fig. 5c plots similar peak variance data as Fig. 5b but now as peak variance increase ($\Delta \sigma^2_t$) compared to the case of $V_{\text{ov}} = 0$ µL.
As mentioned in the experimental section, the sample solvent was chosen the same as the mobile phase modifier, i.e., methanol, to limit the parameter space. The rather strong solvent is however not the most ideal sample solvent and is as a result also not the ideal choice as overfeed solvent. In recent work by Berger [16,17], isopropyl-alcohol was used as the overfeed solvent, but he also observed significant loss in performance when the overfeed volume increased [17]. Using a very apolar solvent, such as hexane or heptane, as overfeed solvent, a reduction in band broadening is expected. Several other studies reported the decrease in band broadening in SFC when an increasingly larger fraction of the sample solvent was replaced by these apolar solvents [6,7,11,13,18]. The poor solubility of most sample compounds in these solvents, in combination with their high volatility however makes them a non-ideal practical alternative as sample solvent. Their use as an overfeed solvent is also limited as these solvents are very volatile and they might not be fully compatible with the fluidic paths in the injector which are designed for reversed phase type solvents. To circumvent these issues, a user defined injection program was implemented where the solvent for the overfeed was first drawn from a sample vial, followed by the drawing of the sample. By drawing a much larger volume (min. 3x the set V_{ov}) of the solvent in the sample loop than actually needed to inject as the overfeed, none of the strong solvent (MeOH) present in the loop is injected in the column. As a result, only a plug of relatively weaker solvent is injected after the sample plug which is still based on the pure methanol solvent. Any remaining hexane in the sample loop is removed in the subsequent wash steps of the autosampler [5]. To limit the number of experiments, a wide range of possible solvent was tested as possible overfeed solvents, including ethanol (EtOH), isopropyl-alcohol (IPA), acetonitrile (ACN), water (H₂O), methanol/water mixtures in different ratios, tetrahydrofuran (THF) and methyl-t-butylether (MTBE), for two V_{inj} and V_{ov} combinations, i.e. V_{inj} = 4µL with V_{ov} = 10µL and V_{inj} = 2.5µL with V_{ov} = 5µL. No difference in the chromatograms was observed for both settings besides the V_{inj} - and V_{ov}-effects discussed earlier. Only for the cases where H₂O was used as a co-solvent, significantly variation was observed among the chromatograms of the triplicate injections. These chromatograms however showed very distorted peaks and baselines. Using the higher alcohols, a small decrease in peak width was observed relative to MeOH, with IPA performing better than EtOH, but at the cost of an additional solvent peak that occurred near the first eluting compound. Using either THF or ACN as overfeed solvent resulted, at least for the presently investigated compounds, in a slightly broader peak. A small improvement, similar to that obtained when using IPA, was observed using MTBE as overfeed solvent, however at the cost of a much broader solvent peak (up to k=0.5). Significantly narrower peaks were however observed when using hexane as an overfeed solvent, without increasing the disturbance of the baseline after the solvent peak relative to the methanol overfeed. It was therefore decided to continue only with hexane as an overfeed solvent. It is however important to notice that hexane is probably not always the best alternative to methanol, as it will depend on the combination of sample solvent, sample compounds, stationary phase and mobile phase. Users are therefore advised to test the most interesting combination of overfeed and sample solvent for their application.
Figure 6a shows how the peak variance increases with increasing overfeed volume when the overfeed solvent is hexane. A very similar behavior is observed as for the methanol overfeed-case in Fig. 5c, however the scale of the Y-axis is 10 times smaller than in Fig. 5c, thus reflecting the much smaller additional band broadening due to the overfeed volume, even for very large values of $V_{ov}$. To illustrate this, Fig. 6b replots the data in Fig. 6a, but now in the same scale as Fig. 5c, showing the very large advantage of using hexane as overfeed solvent. In Fig. 7, an overlay of chromatograms is shown for different $V_{ov}$ with methanol and hexane as the overfeed solvent, where the difference in peak width and height is clearly visible. In the $V_{ov}=0\ \mu$L-case, only a negligible amount of overfeed solvent is injected so here it is normal that almost no difference can be observed. A higher retention time can be observed when hexane is used as the overfeed solvent, in agreement with the fact that the retention in the diluted hexane overfeed plug is higher than in the methanol plug. The peak width could further be reduced when the sample solvent was changed from methanol to a mixture of methanol/isopropyl-alcohol/hexane (15/15/70 volume%) as was previously illustrated in literature [12,13,18] (results not shown). Another observation that can be made in Fig. 7, especially for the last eluting peak, is that the peak shape deteriorates and that some tailing is observed when using MeOH as overfeed solvent. At 0μL overfeed, the USP tailing factor was around 0.95-0.97 for all peaks, however for 10μL methanol overfeed, this increased almost linearly to 1.20 and 1.31 for the second and third peak. The change for the first peak was negligible. For the hexane overfeed, no significant increase in USP tailing factor was observed. This indicates that the solvent mismatch effect of the MeOH overfeed plug is responsible for this peak deformation and that possibly the hexane plug cause some short of focusing effect of the tailing end of the sample plug. Another beneficial aspect of the hexane overfeed could be the reduced viscosity (~0.3mPas for hexane, ~0.6mPas for MeOH) mismatch between the CO$_2$/MeOH mobile phase (~0.11mPas) and the sample+overfeed plug, reducing the possibility of viscous fingering. However, since the flow instabilities that result from viscous fingering are rather random in nature, the chromatograms obtained in the present study would not have had the high reproducibility that was observed in the current study.

3.3 Partial sample injection

Even if there would be no effect of sample solvent mismatch, the variance of the injected peaks would not correspond to the value of an ideal rectangular plug, i.e. $\sigma_V^2 = \frac{V_{inj}^2}{12}$, as the result of the band broadening during sample loading and injection. This was discussed in detail for a flow-through needle type injector in LC in previous experimental work [19] and numerical simulations of the injection process [15]. Figure 8a shows an illustration of the sample plug in the needle at the end of the loading step, based on the simulations performed in [15]. As a consequence of the parabolic flow profile that establishes in the open tubing, the sample compounds in the middle of the tube travelled almost twice the distance as those close to the wall. After the sample is drawn into the loop, the needle needs to move out of the sample vial, back into the needle seat and the flow path needs to be pressurized before injection. During this time (typically 5-10s or more), radial equilibration of the concentration...
profile established during the loading step occurs, resulting in a radially uniform sample plug as shown in Fig. 8b. The peak variance of the sample plug is therefore much larger than for an ideal rectangular plug due to the long dilute tail present. If one could however inject only the first part of the loaded sample, i.e., without the tailing part, a much smaller peak width can be expected, more closely resembling a rectangular injection plug.

This technique is similar to the so-called temporary/timed injection or cut injections presented by Coq. et al. [20] and Samuelson and Fornstedt [21] for use in liquid chromatography to solve the following problem: when a sample loop which is completely filled with sample (overfilled) is switched in line with the mobile phase flow, the solutes present near the wall of the tube will elute much slower than those in center of the loop, causing a long dilute tail (see e.g. Fig. 2 in [22] and Fig. 1a in [21]). By switching the valve before the dilute tail of the sample plug exits the sample loop, an almost perfect rectangular plug can be injected (see Fig. 1b in [21]). The elution behavior from these sample loops was studied in more detail by Samuelson et al. using numerical simulations [23]. In another study, Samuelson and Fornstedt used this technique to inject volumes of 50-900µL, but with an overfilled fixed loop type injector, to improve the determination of adsorption isotherms since the underlying models assumes the injection of perfect rectangular plug [21].

In order to obtain this type of injection for the feed sampler, a similar injection program as for the hexane overfeed was used. Rather than loading a large hexane plug before the sample, a much larger volume of sample (at least 4x larger) was now loaded in the needle and only a small part was injected, with the overfeed volume set to zero. This ensured the injection of only the high concentration part of the sample plug (red zone in Fig. 8b). The downside of this method is that the accuracy of the injected sample amount depends only on the accuracy of the switching time of the injection valve, rather than on the volume metered during sample loading. In addition, the instrument software also ensures that the volume between the needle tip and the injection valve, i.e., that of the needle seat and needle seat capillary, is additionally injected as this precedes the sample plug in the flow path. Since there is always some uncertainty on this volume (e.g. due to the variation in tubing ID of the needle seat capillary), small differences between the true and set volume can occur, which can cause a deviation especially when injecting small sample volumes with no overfeed volume. As this investigate method is not the intended injection method for this injector, it can thus not be expected to meet the requirements in terms of reproducibility and accuracy of a normal sample injector.

To investigate in what range of injection volumes this technique could be advantageous, the peak areas resulting from a wide range of injection volumes (from 0.13 to 10 µL) were determined. Since for all injection volumes the same sample concentration was used, the peak areas were normalized by their injection volume. The results are presented in Fig. 9a. It is clear that, for small injection volumes, a much larger relative peak area is found than for larger injection volumes, clearly indicating that this technique is not very accurate for the small volumes. For the used injection flow rate (1000µl/min) and e.g. a small injection volume of 0.25µL, the injection time for the sample volume is only 15ms, which is in the same order
of the time needed for a valve switch. If there e.g. would be time delay of 5ms on this valve switching, this is very significant for low injection volumes, but becomes negligible for higher injection volumes, hence the decreasing trend observed for $V_{\text{inj}}<1.5\mu\text{L}$. This also has a result on the precision of the injector, as represented by the ±1σ error flags on the data in Fig. 9a. Note that for all other figures, including Fig. 9b, any error flags would not be visible as they are always smaller than the symbol sizes.

For comparative purposes, the peak areas obtained for the injection of the same volumes, but now with an overfeed volume equal to 5 times the injection volume, are overlaid. The choice to have a fixed $V_{\text{ov}}/V_{\text{inj}}$-ratio in these experiments was made because this represents a more appropriate comparison when studying its effects on band broadening, as the peak variance increases with increasing overfeed volume (see Fig. 5). Only the results for the most weakly retained compound are shown, but very similar behavior was found for all compounds. For this series, a plateau value in relative peak area is obtained around $V_{\text{inj}} = 1\mu\text{L}$ corresponding to an overfeed volume of 5 µL. It is clear that, for lower injection volumes (and thus lower overfeed volumes), not the entire sample plug is injected, as a much smaller peak area is obtained. A minimum overfeed volume is thus required, even for small sample volumes, to ensure the entire volume of the sample drawn in the needle is injected. Starting from $V_{\text{inj}} = 2\mu\text{L}$ and higher, the overfeed volume was held constant at 10 µL, which is the maximum set value in the instrument. Since this causes a decrease in $V_{\text{ov}}/V_{\text{inj}}$ for $V_{\text{inj}}>2\mu\text{L}$, a slight downward slope in relative peak area is observed.

Fig. 9b shows the peak variance for both injection modes as a function of the square of the injection volume. For the injections with overfeed, hexane was used as overfeed solvent, i.e., these data points already correspond to the most favorable injections presented in Fig. 6. It can however be seen that the partial sample injection still gives a slight improvement over the hexane overfeed method. The peak variance of the latter series initially increases rather steeply, but this is not only the result of the increase in drawn sample volume, but also because a larger fraction of the drawn sample solvent, which is pure MeOH, is actually injected in the column (cf., the increase in relative peak area with increasing $V_{\text{inj}}$ for the overfeed-case data in Fig. 9a). The partial sample injection thus seems to be an alternative for the hexane overfeed when considering band broadening but suffers from a lack of precision for smaller injection volumes. Similar to the hexane overfeed case with varying overfeed volume (see previous section), the partial sample injection maintains a very symmetrical peak shape, with a USP tailing factor around 1.02-1.07 for $V_{\text{inj}}=1\mu\text{L}$, which decreases linearly to around 0.85-0.95 for the highest injection volumes. The highest peak volumes in fact thus show a slight fronting. Since in this case no focusing effect can occur since no hexane is injected after the sample, cutting off the tailing end of the sample plug (see Fig. 8b) appears to result in a very sharp read end of the sample plug. The broader peak front could be the result of the small volume of methanol which is inherently present in the injector needle seat capillary between the needle tip and injector valve.

### 4. Conclusions
The effect of the different method parameters of the novel feed injector for SFC on band broadening was investigated. The dilution of the sample that occurs during injection results in both a positive and a negative effect on the band broadening. As the sample solvent is diluted with the mobile phase, a more compatible plug of solvent is injected in the column, resulting in a decrease in peak defocusing at the head of the column. On the other hand, the sample compounds are more diluted, and the total volume of the injected peak is increased. Due to these opposing effects, an optimum feed flow rate $F_{\text{feed}}$ exists where a trade-off is made between reduction in the defocusing effect and the increase in peak volume. It was found that this optimal ratio of $F_{\text{feed}}/F$ is independent of the applied flow rate $F$. The optimal $F_{\text{feed}}/F$ depends on the sample compound and decreases with increasing solvent strength dependency of the retention factor with the modifier fraction. The optimal $F_{\text{feed}}$ should thus be chosen based on the most critical pair for which the injection should be optimized.

The overfeed volume injected to ensure full introduction of the sample drawn into the needle causes a steep linear increase in peak variance. The larger the overfeed volume, however, the larger the fraction of the sample drawn into the needle that is finally injected. This implies a compromise between injection accuracy and extra band broadening needs to be made. By using a modified injection method, the overfeed solvent could be changed from the routinely used polar organic solvents (e.g. methanol) that have a high elution strength to a volatile apolar solvent that has properties closer to supercritical CO$_2$ such as hexane. With such a type of overfeed solvent, the increase of the peak variance with increasing overfeed volume was reduced for the investigated compounds and conditions by a factor 10.

Finally, using the partial sample injection method, only the rectangular part of the sample plug in the needle and not the dilute tail, can be injected. This results in an additional gain in performance (decrease in peak variance) compared to the hexane overfeed injection method.

The present study provides a first insight in the behavior of the sample injector. More elaborate investigations will be required under different conditions as this study was limited to a single set of sample compounds and one stationary phase. Although one can expect similar trends for other sample compounds and stationary phases, the absolute values of e.g. the optimal $F_{\text{feed}}/F$ and the amplitude of the effect of $V_{\text{ov}}$ on peak variance will surely be different. In the current study, the volume fraction of modifier (12%) was rather moderate. It can be expected that the observed effects will be more pronounced for separations with a lower fraction of modifier, as the injected solvent plugs will result in an even larger mismatch of the composition of the injected sample plug solvent from that of the mobile phase. In these cases, it is likely that smaller $F_{\text{feed}}/F$-ratios will yield an optimal performance. For separations where a larger fraction of modifier is used at the start, this mismatch will be less pronounced and here larger optimal $F_{\text{feed}}/F$-ratios are expected.

For conditions where the injection solvent/mobile phase mismatch causes significant distortion of the sample plug upon injection, the feed injection technique shows interesting features as it allows to tune the dilution of the sample plug with the mobile phase. It will
therefore depend on the importance of this solvent mismatch on the separation performance, relative to the inherent increase in sample plug volume and the effects of the overfeed plug, whether this injection provides improved performance over the more commonly used fixed loop type injectors in SFC.

Acknowledgements:

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References


[16] T.A. Berger, Reduced plate height of 1.65 on a 20 × 3 mm column packed with 1.8 µm particles in supercritical fluid chromatography (SFC), Chromatographia 82 (2019) 971–974.


Figure Captions:

Figure 1: Basic schematic representation of the injection method. (a) The end of the load step: the needle has drawn the sample and returned to the seat, followed by a compression of the sample loop to the system pressure. (b) The valve switches for injection, allowing the sample and the overfeed to join the mobile phase stream and flow to the column. The orange arc represents the rotor-stator groove. The overfeed solvent is at all times present in the sample needle+loop, the overfeed plug (green) is indicated as the additional injected volume after sample plug, but is thus not different from the solvent present in the rest of the loop. For the investigated cases with an alternative overfeed solvent (see Fig. 6), the overfeed solvent plug is however drawn from a separate sample vial during the custom injection program, see Section 3.2.

Figure 2: (a) Overlay of the chromatogram peaks for progesterone, testosterone and β-estradiol at F=2.5mL/min with different feed speeds: 1000µL/min (blue), 800µL/min (orange), 500µL/min (red), 250µL/min (black) and 100µL/min (green). Zoom-in on (b) progesterone, (c) testosterone and (d) β-estradiol. V_{inj}=2.5µL, V_{ov}=4µL.

Figure 3: (a) Plot of $\sigma^2_t$ as a function of $F_{feed}/F$ at F=2.5mL/min for the three tested compounds: β-estradiol (green triangles), testosterone (orange circles) and progesterone (blue squares). (b) Plot of the normalized peak variances ($\sigma^2_t / \sigma^2_{t,min}$) as a function of $F_{feed}/F$ for β-estradiol at three different flow rates: F=1.25mL/min (open green triangles), F=2.5mL/min (open orange circles); F=3.75mL/min (open blue squares).

Figure 4: Plot of the retention factor as a function of MeOH fraction at F=2.5 mL/min and $F_{feed}$=1000 µL/min for the three tested compounds (β-estradiol (green triangles), testosterone (orange circles) and progesterone (blue squares)). Equations represent values obtained after fitting with the linear solvent strength model: $k = k_0 e^{-S \varphi}$ with $\varphi$ the set volumetric solvent fraction of modifier (MeOH) in the mobile phase.

Figure 5: Plot of (a) the normalized peak area, (b) the normalized peak variances and (c) the peak variance increase as a function of overfeed volume for the three tested compounds, i.e. β-estradiol (green triangles), testosterone (orange circles) and progesterone (blue squares), when using methanol as overfeed solvent at F=1.25mL/min (a-b) or F=2.50mL/min (c) and $F_{feed}$=1000µL/min, $V_{inj}$=1.25µL (a-b) and $V_{inj}$ = 5µL (c).

Figure 6: (a) Plot of the peak variance increase as a function of overfeed volume for the three tested compounds and (b) replot of the data in the same scale as Fig. 5c, for β-estradiol (green triangles), testosterone (orange circles) and progesterone (blue squares) with hexane as overfeed solvent at F=2.5mL/min and $F_{feed}$=1000µL/min, $V_{inj}$=5.0µL.

Figure 7: Overlay of the chromatogram peaks when using hexane (orange) or methanol (blue) as overfeed solvent with an overfeed volume of (a) 0µL, (b) 5µL and (c) 10µL, F=2.5mL/min and $F_{feed}$=1000µL/min, $V_{inj}$=2.5µL.

Figure 8: Simulated species profiles in a straight sample needle (a) after sample uptake and (b) after a holding time of 5s, where the red color corresponds to areas of high concentrations and blue to low. ID=200µm, $D_m$= $1\times10^{-9}$ m²/s, sampling loading flow rate of 100µL/min and $V_{inj}$=5 µL. Figure adapted from results obtained in [13].
**Figure 9:** Plot of **(a)** the normalized peak area of the progesterone peak (k=0.7), as a function of injection volume and **(b)** the peak variance as a function of injection volume squared, using either a partial sample injection (orange squares) or a fixed $V_{ov}/V_{inj}=5$ ratio (blue diamonds) with hexane as the overfeed solvent at $F=2.5\text{mL/min}$, $F_{feed}=1000\mu\text{L/min}$.
Kim Vanderlinden: Methodology, Validation, Formal analysis, Investigation, Visualization, Writing - original draft, Data Curation.

Gert Desmet: Conceptualization, Writing - Review & Editing

Ken Broeckhoven: Methodology, Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - Review & Editing, Visualization, Resources, Supervision, Funding acquisition
Figure 1:
Figure 3:

(a) 

(b)
Figure 4:

- $y = 7.9997e^{-9.134x}$
  $R^2 = 0.9901$

- $y = 4.4457e^{-7.863x}$
  $R^2 = 0.9903$

- $y = 1.3784e^{-5.658x}$
  $R^2 = 0.9982$
Figure 5:

(a) 

$M_0$

$M_{0,v_0} = 10 \mu L$

$V_{ov} (\mu L)$

$\beta$-estradiol ($k=2.7$)

testosterone ($k=1.8$)

progesterone ($k=0.7$)

(b) 

$\sigma_t^2$

$\sigma_{t,v_0}^2 = 0 \mu L$

$V_{ov} (\mu L)$

(c) 

$\Delta \sigma_t^2 (\text{min}^2)$

$V_{ov} (\mu L)$
Figure 7:

(a) $V_{ov} = 0 \mu L$

(b) $V_{ov} = 5 \mu L$

(c) $V_{ov} = 10 \mu L$

MeOH, hexane
Figure 8:

- Sample load

(a) Dilute tail

(b) Straight plug
Figure 9:

(a) Partial sample injection

Fixed $V_{ov}/V_{inj} = 5$

(b) $\sigma_t^2 (\min^2)$ vs. $V_{inj}^2 (\mu L^2)$
Table 1: Overview of the optimum $F_{\text{feed}}/F$-ratios of the different investigation sample compounds for different $F$ and $V_{\text{inj}}$.

<table>
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<tr>
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<th>Injection volume $V_{\text{inj}}$</th>
<th>F (mL/min)</th>
<th>5µL</th>
<th>2.5µL</th>
<th>1.25µL</th>
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<tr>
<td><strong>Progesterone</strong> (k = 0.7)</td>
<td>3.75</td>
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<tr>
<td><strong>Testosterone</strong> (k = 1.8)</td>
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<tr>
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<td>2.5</td>
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<tr>
<td><strong>β-estradiol</strong> (k = 2.7)</td>
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