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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

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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_{feed}, the total volume in which the sample is contained increases with a factor $(F_{feed}+F)/F_{feed}$. In addition, to ensure that all of the loaded sample is injected on the column, an additional overfeed volume (V_{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_{feed}/F-ratio, V_{ov}, overfeed solvent etc. under SFC conditions. It was found that an optimal F_{feed}/F exists which is independent of F and decreases with increasing solvent strength dependency of the sample compound. Decreasing V_{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 CO_2-based mobile phase. Further reduction of the band broadening could be achieved by applying partial sample injections.

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 CO_2), 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 (CO_2 + 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 CO_2 stream and modifier as is the case in analytical SFC instrumentation, but the injection is made in the modifier-stream, before mixing with the CO_2 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

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88 mobile phase flow towards the column. The injector pump the pushes, or 'feeds' the sample
89 volume, together with a certain overfeed volume of additional solvent present in the
90 needle/sample loop taken from a dedicated solvent bottle, into the mobile phase flow at a
91 set flow rate (max. 1 ml/min). This additionally injected overfeed volume (V_{OV}) is to ensure
92 that the full sample volume accurately metered during sample drawing is injected in the
93 system. Fig. 1 provides a basic schematic representation of the injection method. Fig. 1a
94 represents the end of the load step during which the sample is drawn into the needle,
95 returned to the needle seat and followed compression of the sample loop. Fig. 1b represents
96 the valve position during injection, when the sample and the overfeed are joined with the
97 mobile phase stream. A detailed drawing of the entire injection module (including e.g. wash
98 steps), can be found in the manual of the instrument [5].

99 In SFC, regardless of the type of injector, the sample solvent (usually an organic solvent) is
100 always significantly different from the mobile phase (mainly compressed CO_2). Whereas the
101 mobile phase is rather apolar, the typically used sample solvents that have a high solubility
102 for the sample compounds have a much higher elution strength than the mobile phase,
103 causing a mismatch in retention at the head of the column when the sample is injected [6-
104 10]. In addition, the sample solvent itself can interact with the stationary phase, causing a
105 disturbance in the adsorbed layer of mobile phase modifier (often MeOH in SFC). All these
106 effects can cause significant distortion of the sample band, causing significantly additional
107 band broadening or even strong peak deformation (tailing/fronting) of the peaks. The effect
108 of sample solvent in SFC was investigated in detail by Desfontaine et al. for a wide range of
109 sample compounds, sample solvents and stationary phases [11]. They concluded that the
110 sample solvent effect depends not only on the sample solvent but can also vary significantly
111 for different sample compounds or stationary phases. In a combined experimental and
112 modeling study, Enmark et al. found that (1) solvent strength mismatch is the main reason for
113 peak distortion, (2) the adsorption of MeOH to the stationary phase increases the band
114 broadening and (3) viscous fingering gives additional broadening of the solute bands [2].

115 The use of a feed injector partially alleviates the effects of solvent mismatch between the
116 mobile phase and the sample solvent, as during the injection the sample is mixed with the
117 mobile phase stream. As a result, the sample is diluted proportional to the ratio of the mobile
118 phase flow rate F and the feed flow rate F_{feed}. Taking for example a simplified case (ideal plugs
119 and incompressible flow) where the sample solvent is the same as the mobile phase modifier
120 which is set at 10% (volume-based) at the start of the method. Since $F_{feed}=F$, the sample
121 solvent will be diluted to 55% (v/v) modifier and 45% (v/v) CO_2, rather than pure (100%)
122 solvent. For $F_{feed}=F/3$, the sample will already be diluted to 32.5% (v/v) modifier. As a result,
123 a lower F_{feed} leads to a more "compatible" sample solvent. Following a similar train of thought,
124 also effects of viscosity mismatch between sample plug and mobile phase can be reduced.
125 The inherent downside of this dilution is of course that also the sample concentration is
126 decreased as the total sample volume is increased proportional to F/F_{feed} (in fact it increases
127 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_{feed}, V_{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 β-estradiol (>98%) were purchased from Sigma-Aldrich (Overijse, Belgium). The analytes were dissolved at a concentration of 100 µg/mL in pure MeOH. For the measurements where also very low injection volumes were investigated, a concentration of 500 µg/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 µm 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 µL) and a 2 µL SFC flow cell (3 mm path length) respectively. Data was recorded at a wavelength of 254 nm (progesterone) and 210 nm (testosterone, β-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 µL, while the injection flow rate (feed speed) was varied between 100 – 1000 µL/min. MeOH or hexane were used as overfeed solvent and the overfeed volume (V_{ov}) was either fixed at 4 µL or varied between 0 – 10 µL. All injections were performed in triplicate and the average value was reported in all figures. With the exception

191   of the data presented in Fig. 9, in all other cases the +- 1σ error bars were smaller than the
192   symbols used in the plots and were therefore omitted. To determine peak variances, the
193   method of moments was used.

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

205   3. Results and Discussion

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

211

212   3.1 Effect of feed flow rate on peak width

213   In a first set of experiments, a series of injections were performed with varying feed flow rate
214   F_{feed}, but with at a fixed mobile phase flow rate F. The experiments were performed at three
215   different fixed F (1.25 mL/min, 2.5 mL/min and 3.75 mL/min) and three different V_{inj} (1.25 μL,
216   2.5 μL and 5 μL), with a fixed $V_{ov} = 4\mu L$.

217   Figure 2a shows example chromatograms at F=2.5ml/min for a selection of injections
218   performed with the different F_{feed}-values that were investigated. The chromatograms clearly
219   show that the peak widths and heights vary with F_{feed}, but also that the highest and narrowest
220   peaks are not obtained for the highest F_{feed} of 1000 $\mu L/min$, which corresponds to the least
221   dilution/smallest volume of the peak. To look at this in more detail, Figs. 2b-d show a zoom-
222   in on each of the three different peaks. For the first eluting compound (progesterone), a feed
223   flow rate of 500 $\mu L/min$ (red) seems to yield the highest and narrowest peak, whereas for the
224   second (testosterone) equally high and narrow peaks are observed for 250 and 500 $\mu L/min$.
225   The chromatograms of the last eluting compound clearly show that 250 $\mu L/min$ yields the
226   highest and narrowest peak. In addition, a clear trend in the location of the peak apices is
227   observed: the lower F_{feed} the later the apex. This is not surprising, as for stronger dilution of

228 the sample plug (lower F_{feed}), the sample enters the column in a weaker solvent and is thus
229 initially more strongly retained, yielding higher retention times. The lower feed flow rate also
230 causes a small delay of the timing with which the mean of the sample plug enters the column
231 due to increased plug length. The observed delays were however larger than can be expected
232 from this effect alone.

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

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

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

262 It was already clear from Fig. 3a and can also be seen in Table 1 that the optimal F_{feed}/F-ratio
263 depends on the sample compound, where the more retained compounds appear to require
264 a stronger dilution of the sample plug (lower optimal F_{feed}/F). To better understand this
265 behavior, the retention factor of the three compounds was measured as a function of the
266 modifier fraction in the mobile phase and fitted according to the linear solvent strength model

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267 [14]. Figure 4 shows that the investigated compounds with a higher retention factor also have
268 a much larger solvent strength dependency (S-factor). Previous studies showed that solvent
269 strength mismatch is one the main reasons for peak distortions [2] and this mismatch
270 decreases for lower values of F_{feed}/F. It thus seems that the S-factor mainly determines the
271 optimal F_{feed}/F-ratio since larger S-value result in a larger relative difference of k in the sample
272 solvent plug when in enters the column and k in the normal mobile phase, for a given
273 difference in composition.

3.2 Effect of overfeed volume and solvent

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

291 Fig. 5b plots the corresponding normalized peak variance as a function of V_{ov}, using the peak
292 variance at V_{ov} = 0 µL as the normalization factor as this corresponds to the smallest peak
293 width. A strong linear increase in peak width can be observed with increasing overfeed
294 volume, reaching an increase in peak variance by a factor of 4 (i.e., a doubling of the peak
295 width) for the largest V_{ov} of 10µL. Looking in more detail, a factor of 5 is found for
296 progesterone and a factor around 3.75 for the two other compounds. This is since the early
297 eluting progesterone has the narrowest peak width and thus the smallest $\sigma^2_{t,Vov=0µL}$ used for
298 normalization. The absolute increase in σ^2_t is the largest for β-estradiol (see also Fig. 5c), for
299 the same reason as why it has the lowest optimal F_{feed}/F ratio discussed in the previous
300 paragraph, i.e. because it has the strongest dependency of retention modifier fraction. The
301 combined effect of the increase in injected sample amount (Fig. 5a) and increase in peak
302 width (Fig. 5b) however results in a maximum sensitivity (highest peak height) for an overfeed
303 volume of around 1.25 - 1.5 µL. For an easy comparison (see next paragraph), Fig. 5c plots
304 similar peak variance data as Fig. 5b but now as peak variance increase ($\Delta\sigma_t^2$) compared to
305 the case of V_{ov} = 0 µL.

9

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 (H_2O), 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 H_2O 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.

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347 Figure 6a shows how the peak variance increases with increasing overfeed volume when the
348 overfeed solvent is hexane. A very similar behavior is observed as for the methanol overfeed-
349 case in Fig. 5c, however the scale of the Y-axis is 10 times smaller than in Fig. 5c, thus reflecting
350 the much smaller additional band broadening due to the overfeed volume, even for very large
351 values of V_{ov}. To illustrate this, Fig. 6b replots the data in Fig. 6a, but now in the same scale as
352 Fig. 5c, showing the very large advantage of using hexane as overfeed solvent. In Fig. 7, an
353 overlay of chromatograms is shown for different V_{ov} with methanol and hexane as the
354 overfeed solvent, where the difference in peak width and height is clearly visible. In the $V_{ov} =$
355 0 μL-case, only a negligible amount of overfeed solvent is injected so here it is normal that
356 almost no difference can be observed. A higher retention time can be observed when hexane
357 is used as the overfeed solvent, in agreement with the fact that the retention in the diluted
358 hexane overfeed plug is higher than in the methanol plug. The peak width could further be
359 reduced when the sample solvent was changed from methanol to a mixture of methanol/
360 isopropyl-alcohol/hexane (15/15/70 volume%) as was previously illustrated in literature
361 [12,13,18] (results not shown). Another observation that can be made in Fig. 7, especially for
362 the last eluting peak, is that the peak shape deteriorates and that some tailing is observed
363 when using MeOH as overfeed solvent. At 0μL overfeed, the USP tailing factor was around
364 0.95-0.97 for all peaks, however for 10μL methanol overfeed, this increased almost linearly
365 to 1.20 and 1.31 for the second and third peak. The change for the first peak was negligible.
366 For the hexane overfeed, no significant increase in USP tailing factor was observed. This
367 indicates that the solvent mismatch effect of the MeOH overfeed plug is responsible for this
368 peak deformation and that possibly the hexane plug cause some short of focusing effect of
369 the tailing end of the sample plug. Another beneficial aspect of the hexane overfeed could be
370 the reduced viscosity (~0.3mPas for hexane, ~0.6mPas for MeOH) mismatch between the
371 CO_2/MeOH mobile phase (~0.11mPas) and the sample+overfeed plug, reducing the possibility
372 of viscous fingering. However, since the flow instabilities that result from viscous fingering are
373 rather random in nature, the chromatograms obtained in the present study would not have
374 had the high reproducibility that was observed in the current study.

375 **3.3 Partial sample injection**

376 Even if there would be no effect of sample solvent mismatch, the variance of the injected
377 peaks would not correspond to the value of an ideal rectangular plug, i.e. $\sigma_V^2 = V_{inj}^2/12$, as the
378 result of the band broadening during sample loading and injection. This was discussed in
379 detail for a flow-through needle type injector in LC in previous experimental work [19] and
380 numerical simulations of the injection process [15]. Figure 8a shows an illustration of the
381 sample plug in the needle at the end of the loading step, based on the simulations performed
382 in [15]. As a consequence of the parabolic flow profile that establishes in the open tubing, the
383 sample compounds in the middle of the tube travelled almost twice the distance as those
384 close to the wall. After the sample is drawn into the loop, the needle needs to move out of
385 the sample vial, back into the needle seat and the flow path needs to be pressurized before
386 injection. During this time (typically 5-10s or more), radial equilibration of the concentration

387 profile established during the loading step occurs, resulting in a radially uniform sample plug
388 as shown in Fig. 8b. The peak variance of the sample plug is therefore much larger than for
389 an ideal rectangular plug due to the long dilute tail present. If one could however inject only
390 the first part of the loaded sample, i.e., without the tailing part, a much smaller peak width
391 can be expected, more closely resembling a rectangular injection plug.

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

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

420 To investigate in what range of injection volumes this technique could be advantageous, the
421 peak areas resulting from a wide range of injection volumes (from 0.13 to 10 µL) were
422 determined. Since for all injection volumes the same sample concentration was used, the
423 peak areas were normalized by their injection volume. The results are presented in Fig. 9a. It
424 is clear that, for small injection volumes, a much larger relative peak area is found than for
425 larger injection volumes, clearly indicating that this technique is not very accurate for the
426 small volumes. For the used injection flow rate (1000µl/min) and e.g. a small injection volume
427 of 0.25µL, the injection time for the sample volume is only 15ms, which is in the same order

428 of the time needed for a valve switch. If there e.g. would be time delay of 5ms on this valve
429 switching, this is very significant for low injection volumes, but becomes negligible for higher
430 injection volumes, hence the decreasing trend observed for $V_{inj}<1.5\mu L$. This also has a result
431 on the precision of the injector, as represented by the $\pm 1\sigma$ error flags on the data in Fig. 9a.
432 Note that for all other figures, including Fig. 9b, any error flags would not be visible as they
433 are always smaller than the symbol sizes.

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

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

467

4. Conclusions

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

481 The overfeed volume injected to ensure full introduction of the sample drawn into the needle
482 causes a steep linear increase in peak variance. The larger the overfeed volume, however, the
483 larger the fraction of the sample drawn into the needle that is finally injected. This implies a
484 compromise between injection accuracy and extra band broadening needs to be made. By
485 using a modified injection method, the overfeed solvent could be changed from the routinely
486 used polar organic solvents (e.g. methanol) that have a high elution strength to a volatile
487 apolar solvent that has properties closer to supercritical CO_2 such as hexane. With such a type
488 of overfeed solvent, the increase of the peak variance with increasing overfeed volume was
489 reduced for the investigated compounds and conditions by a factor 10.

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

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

505 For conditions where the injection solvent/mobile phase mismatch causes significant
506 distortion of the sample plug upon injection, the feed injection technique shows interesting
507 features as it allows to tune the dilution of the sample plug with the mobile phase. It will

508 therefore depend on the importance of this solvent mismatch on the separation
509 performance, relative to the inherent increase in sample plug volume and the effects of the
510 overfeed plug, whether this injection provides improved performance over the more
511 commonly used fixed loop type injectors in SFC.

512

513 **Acknowledgements:**

515

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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 σ^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: $k = k_0 \cdot e^{-S \cdot \varphi}$ with φ 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= 1×10^{-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=2.5mL/min, $F_{feed}=1000\mu L/min$.

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
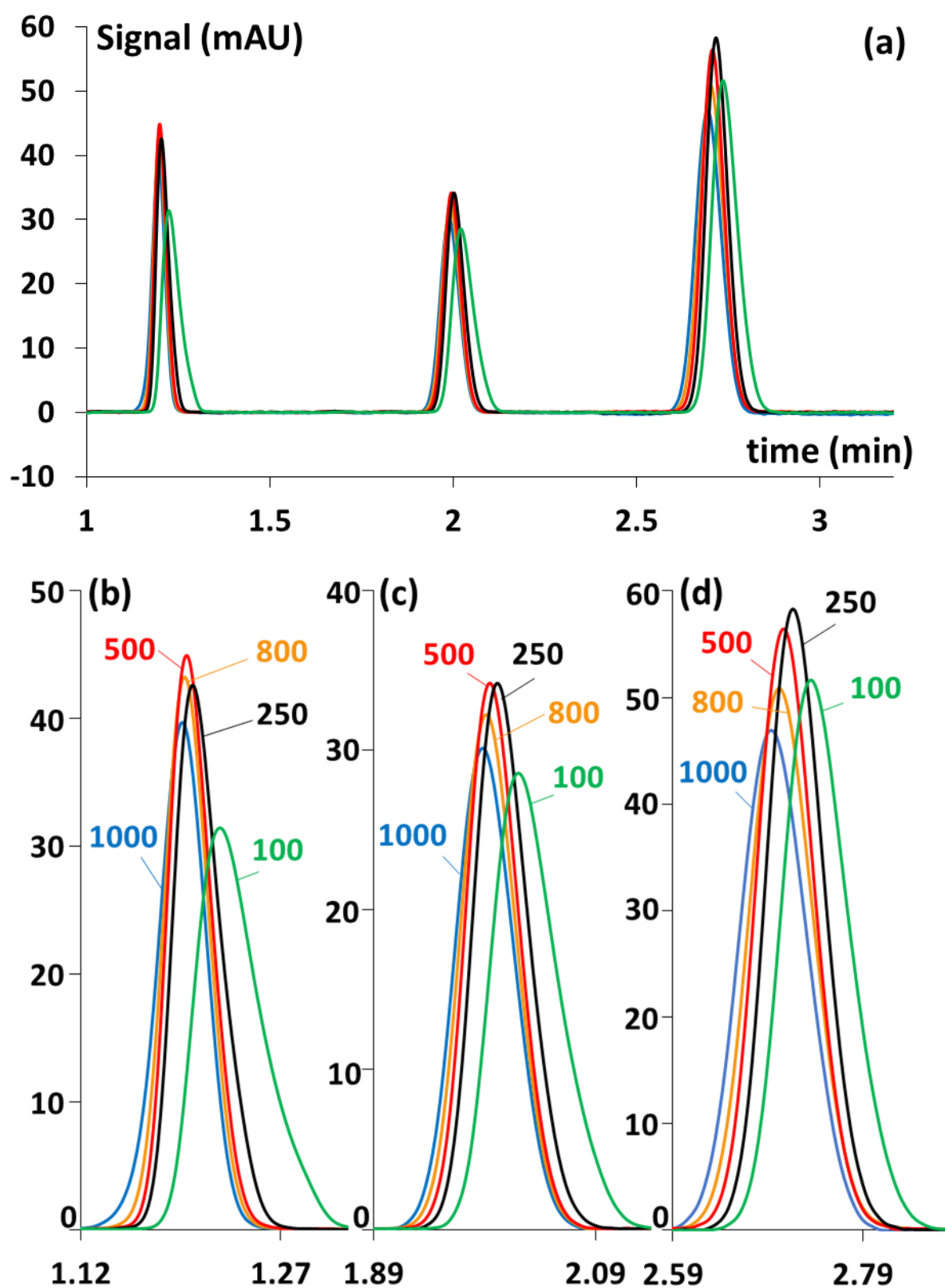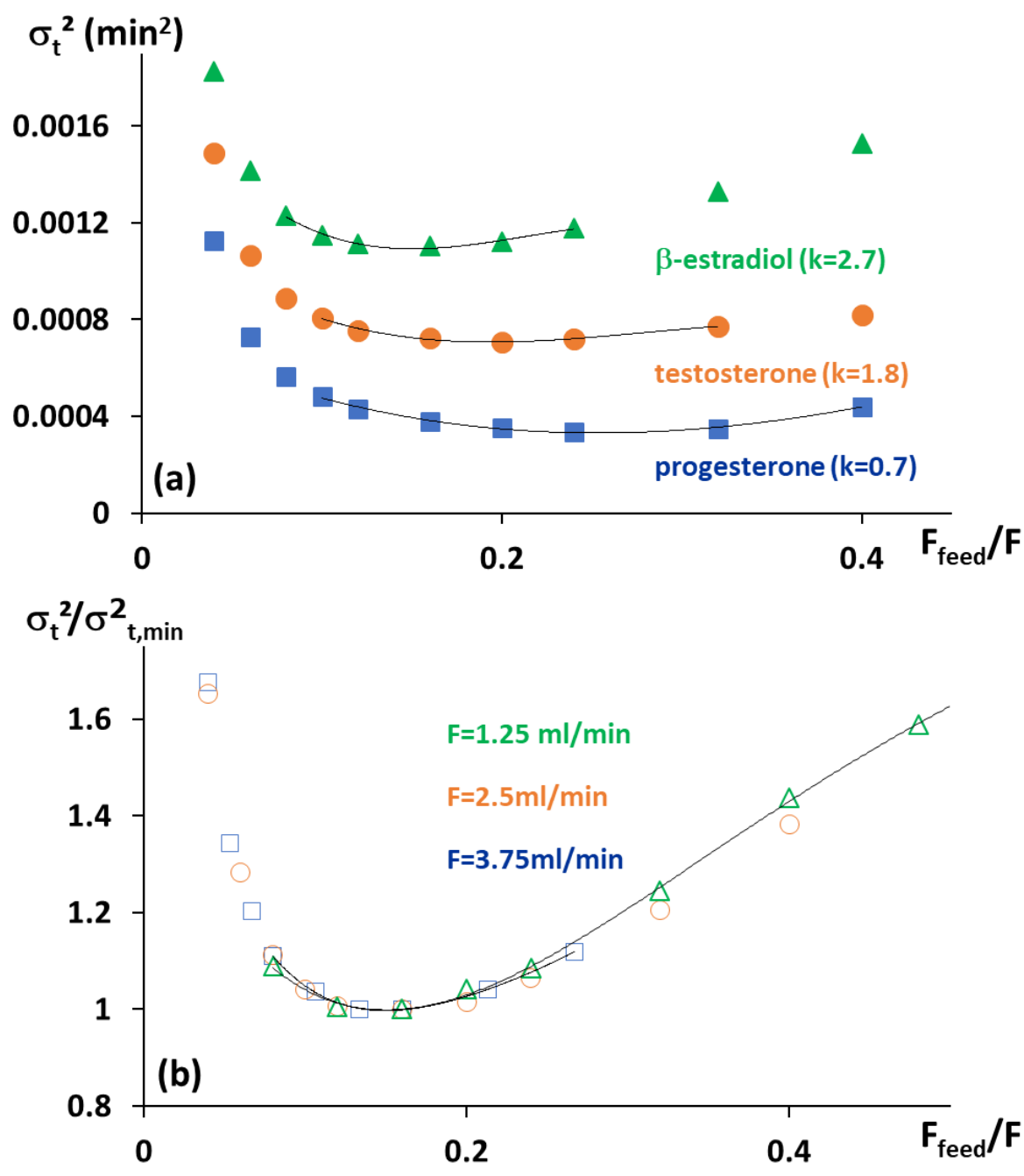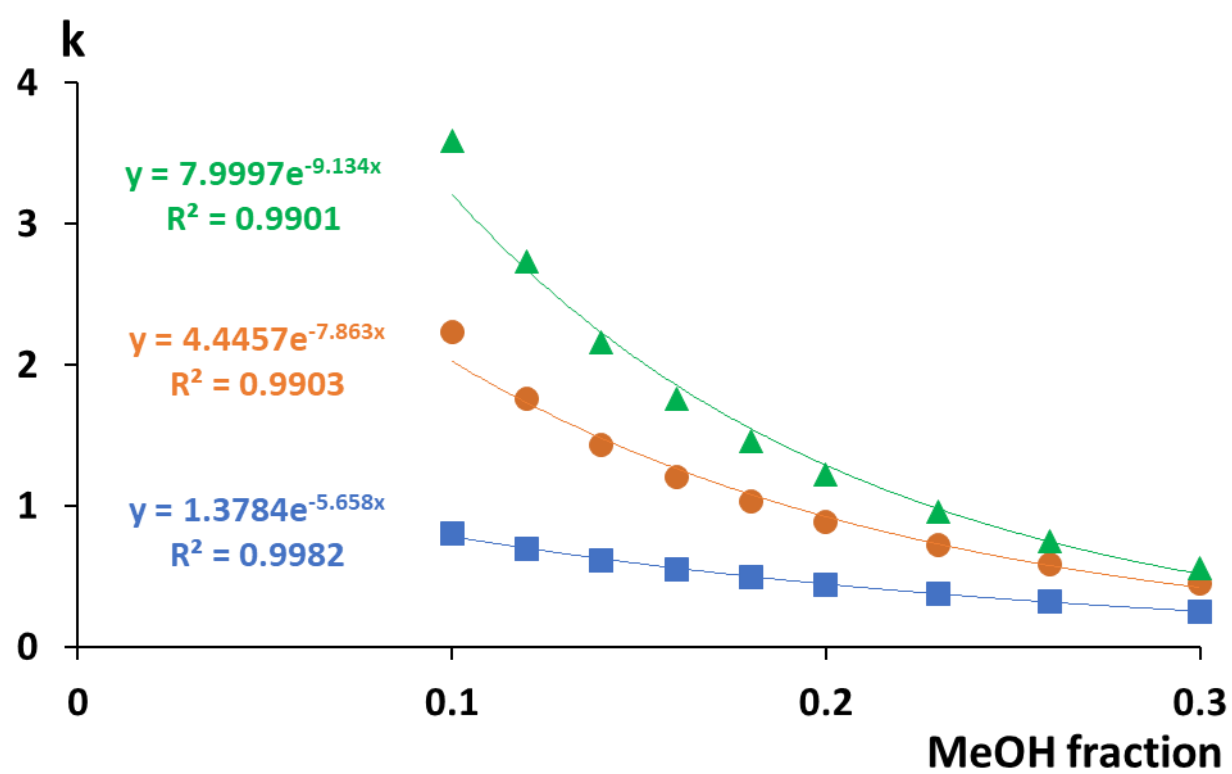
Figure 1:

Figure 2:

Figure 3:

(a) Plot of σ_t^2 (min²) versus F_{feed}/F showing β-estradiol (k=2.7), testosterone (k=1.8), and progesterone (k=0.7).

(b) Plot of $\sigma_t^2/\sigma_{t,min}^2$ versus F_{feed}/F for F=1.25 ml/min, F=2.5 ml/min, and F=3.75 ml/min.

Figure 4:

Figure showing k versus MeOH fraction with three exponential fit curves:

$y = 7.9997e^{-9.134x}$, $R^2 = 0.9901$ (green triangles)

$y = 4.4457e^{-7.863x}$, $R^2 = 0.9903$ (orange circles)

$y = 1.3784e^{-5.658x}$, $R^2 = 0.9982$ (blue squares)
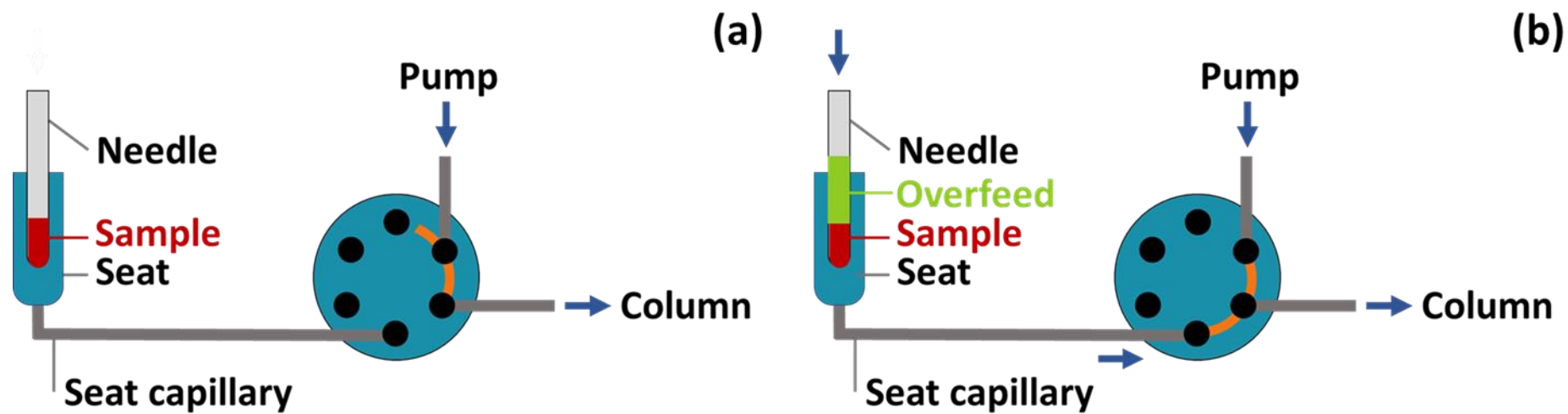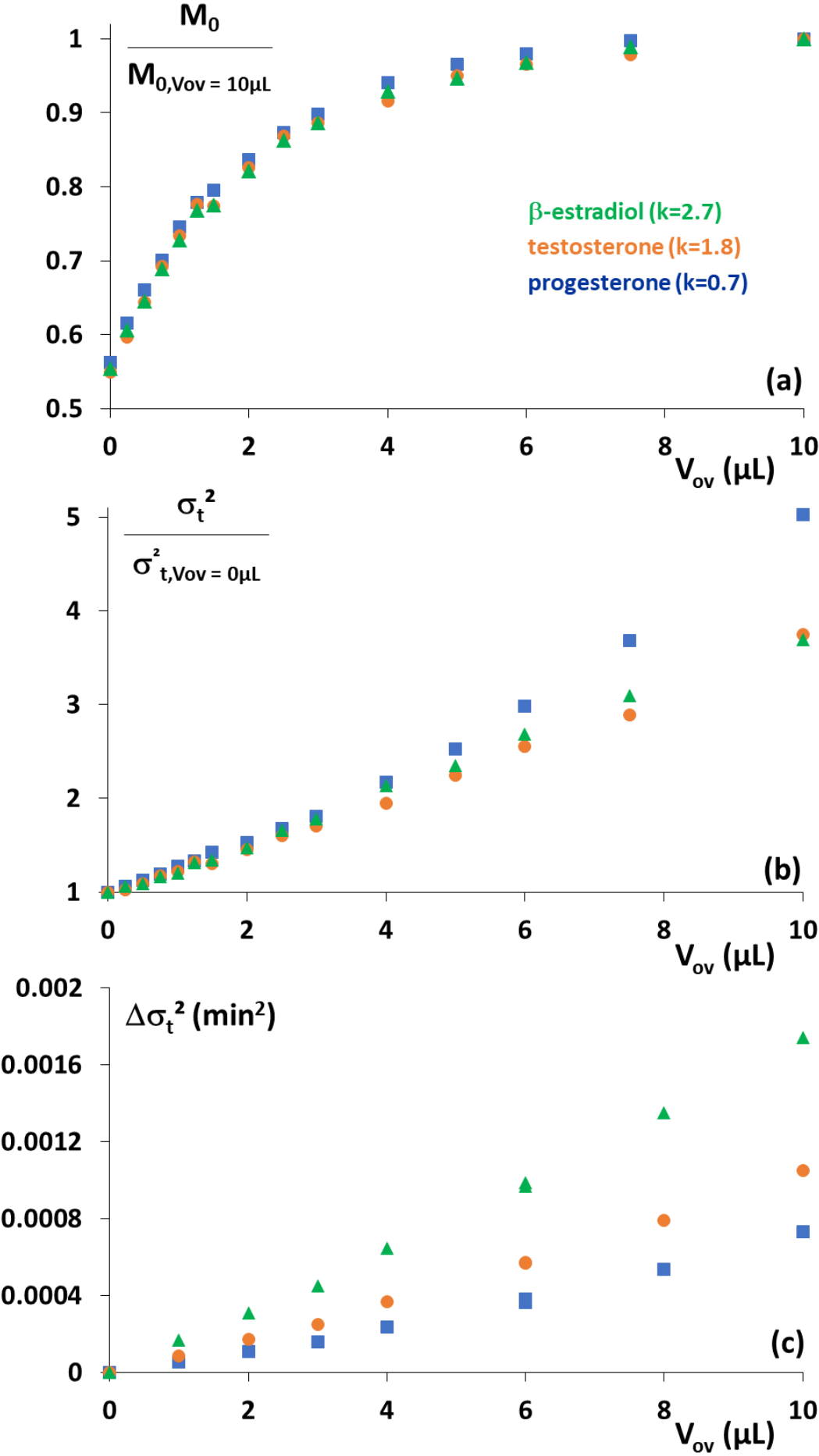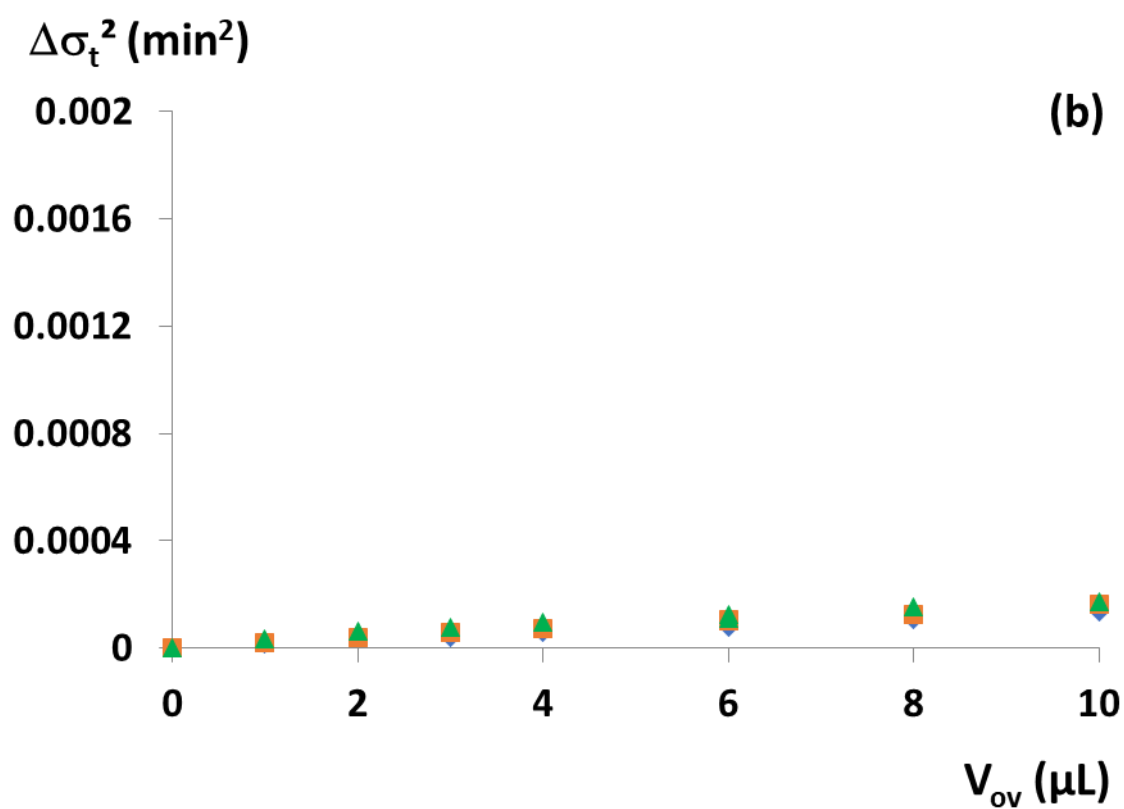
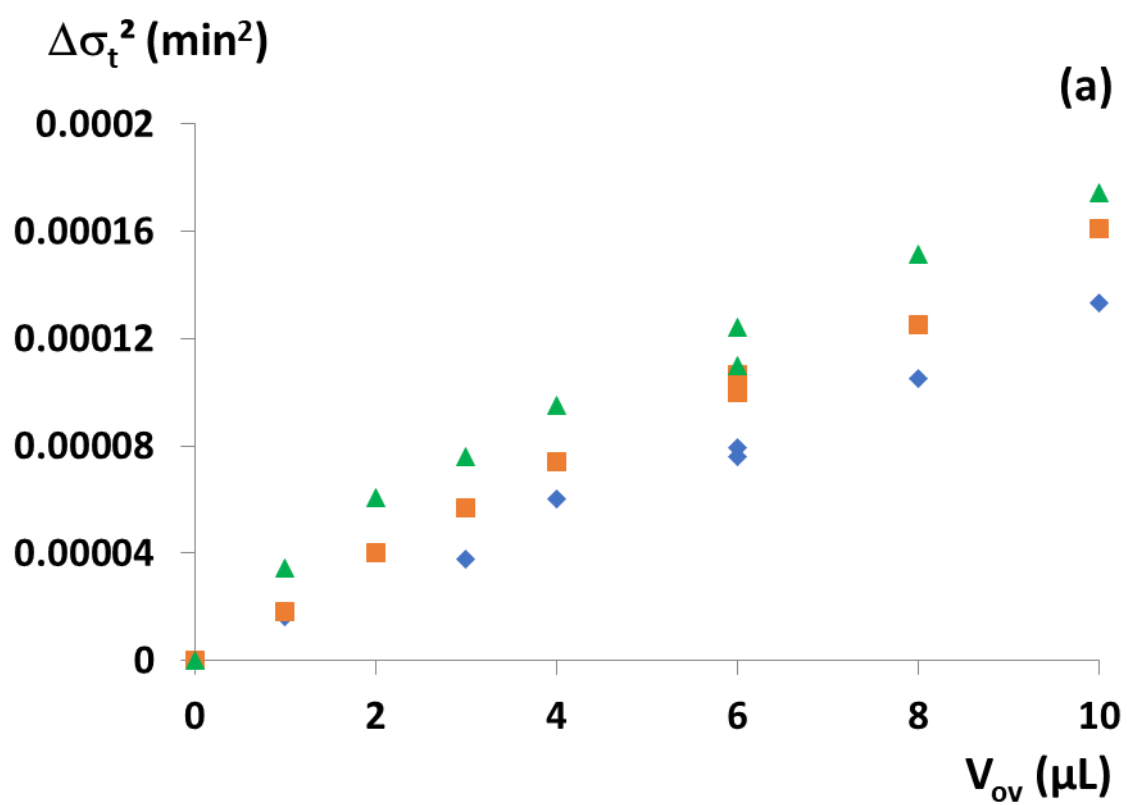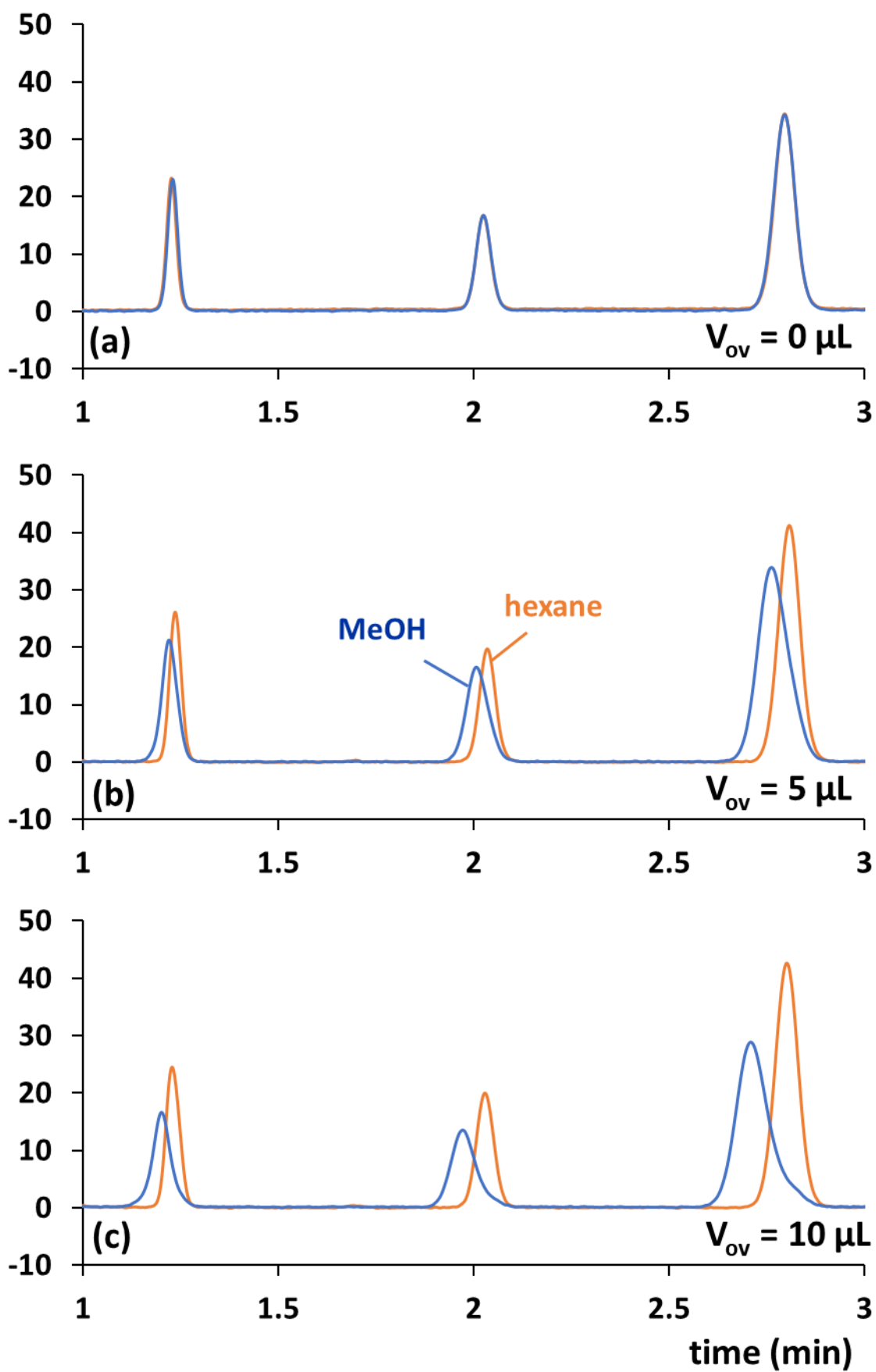**Figure 5:**

Figure 6:
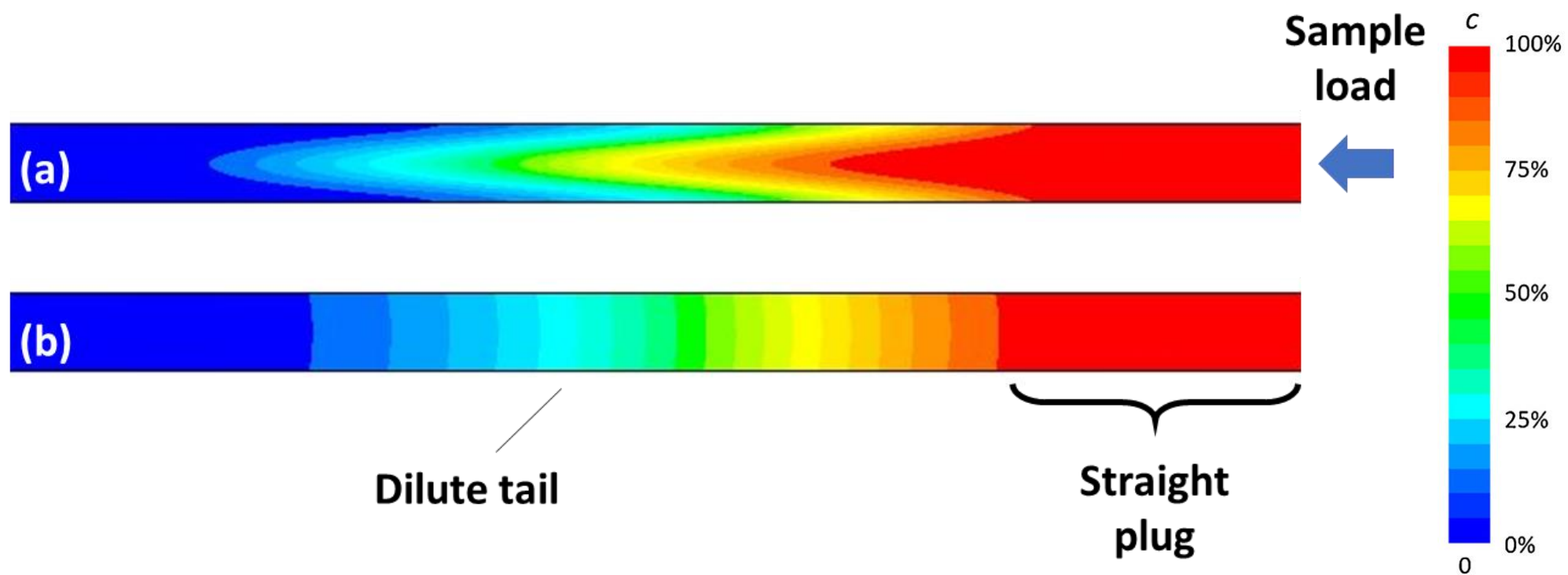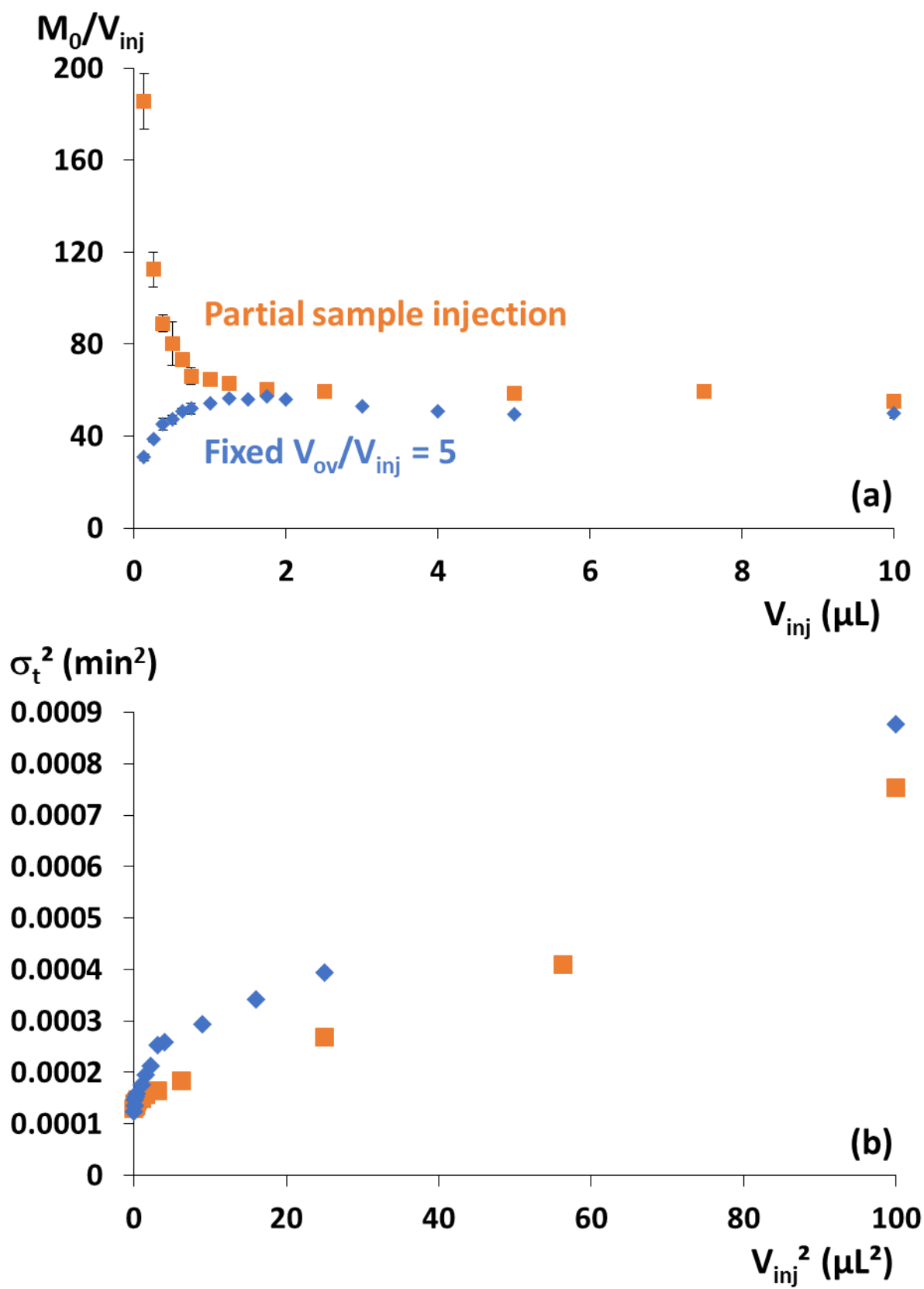
Figure 7:

Figure 8:

Figure 9:

Table 1: Overview of the optimum F_{feed}/F-ratios of the different investigation sample compounds for different F and V_{inj}.

	F (mL/min)	Injection volume V_{inj}		
		5µL	2.5µL	1.25µL
Progesterone (k = 0.7)	3.75	0.27		
	2.5	0.26	0.23	
	1.25	0.265	0.22	0.167
Testosterone (k = 1.8)	3.75	0.195		
	2.5	0.195	0.16	
	1.25	0.18	0.17	0.137
β-estradiol (k = 2.7)	3.75	0.16		
	2.5	0.155	0.16	
	1.25	0.15	0.14	0.125