Practical Improvements in SFC-MS for Achiral Purification

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At AstraZeneca R&D Mölndal, Sweden, many compounds are purified every day. It is therefore necessary to use a process plate format with generic methods, allowing for fast analysis and an easy scale up for preparative scale purification. Traditionally, all purifications were performed using RPLC-MS, but since 2012 approximately 50% of the compounds are purified using SFC-MS. Initially SFC-MS was more time consuming than RPLC-MS, as each separation needed to be optimised in several steps before purification.

In this paper, we describe how analysis in SFC-MS has been optimised with regard to both sensitivity and robustness. We show a simple scale up procedure in which focused gradients for purification are predicted from the retention time in analysis. Finally, we discuss how recovery in SFC purification has been increased from 90% to 95% by using sample sandwiching injection.

Drug discovery is challenging and the search for new medicines needs to become more efficient. To deliver high quality compounds for testing, it is of great importance to ensure a time efficient way to purify compounds in large numbers. In our laboratory at AstraZeneca R&D Mölndal, RPLC-MS and SFC-MS are used as complementary techniques. Every year roughly 3000 achiral compounds are purified, which limits the time for method optimisation, therefore scale-up methods need to be generic. In addition all crude samples are delivered as DMSO solutions and this has to be considered. DMSO can give injection solvent effects in both SFC and RPLC, and it is also retained in SFC, which will put constraints on scale-up.

When implementing SFC in 2012 great efforts were made to optimise a purification method for each individual compound. This was very time consuming and therefore the majority of the compounds were purified using RPLC. This has been overcome by development of an easy scale-up methodology from a generic analytical gradient to a focused gradient in preparative scale. It was also observed that the recovery was lower in SFC than in RPLC, and that the difference was even higher for small amounts. Compounds less than 20 mg in scale were therefore purified using RPLC by default. Using an alternative injection technique has now circumvented this limitation.

Experimental

All SFC chromatography was performed using CO_2 with MeOH (DEA or NH_3) at 120 bar, 40°C. Initially, the analytical data was acquired using a Waters X5 SFC MS instrument that can be operated in parallel mode using five columns. The method was a 10-40% modifier gradient (3 min). The flow rate was 4 ml/min for single mode and 20 ml/min for parallel mode. The make-up flow for the MS was 0.2 ml/min of MeOH/H_2O (95/5 v/v) with 10 mM NH_4HCO_2.

To improve performance and minimise downtime, the Waters X5 SFC MS system was rebuilt into a single mode system. Tier 1 screen was performed using Waters Viridis 2-EP and Phenomenex Luna HILIC columns (5 µm, 250 x 4.6 mm).

A Waters Acquity UPC² MS, equipped with a PDA detector and a column oven with 4 positions, later replaced the analytical X5 system. The method was changed to a 5-50% modifier gradient (4 min, 2.5 ml/min). Tier 1 screen was performed using Waters BEH 2-EP (3.5 µm), Waters BEH (3.5 µm) and Phenomenex Luna HILIC (3 µm) columns (100 x 3 mm). The make-up flow for the MS was 0.4 ml/min.

Preparative separations were performed using Waters Prep 100 SFC MS systems. In the focused gradients the modifier percentage was held constant for one minute and then increased with 5% in 6 min (100 g/min). The scale up and recovery experiments were performed using either a Waters Viridis 2-EP or Waters BEH 2-EP column (5 µm, 250 x 30 mm).

Recovery was measured by evaporating the fractions in tare weighed tubes using a Genevac HT12 vacuum centrifuge over night.

The RPLC analysis was performed on a Waters Acquity UPLC SQD MS system using Waters BEH C18 column (1.7 µm, 50 x 2.1 mm) and a generic gradient (2-94% ACN in 2.5 min, 1 ml/min, 0.2% NH_3 v/v, 45°C).

Results and Discussion

When SFC-MS was implemented in our laboratory, analytical separations were performed on a Waters X5 SFC MS system using five columns in parallel. The columns were connected to separate UV detectors, but then the flows were combined before the MS giving a single MS chromatogram for all columns. Parallel mode ensured fast screening of five columns, but the combined MS trace resulted in a complicated evaluation and often, the most promising method had to be verified in single mode run.

The screening method initially used was a gradient running from 10% to 40% MeOH using DEA (0.5% v/v) as basic additive in the modifier. DEA is known to suppress ionisation of many compounds, and this in combination with the parallel mode made the MS spectra almost impossible to interpret when running crude samples. Therefore the basic additive was changed to 20 mM NH_3 (stock solution 2 M NH_3 in MeOH) [1].

Running the system in parallel mode also resulted in substantial hardware/software problems with leaking check valves and recurring communication problems. The X5 system was rebuilt into a single mode system. The chromatograms became easier to evaluate and the downtime of the system was dramatically decreased, but the screening became more time consuming and therefore, only two columns (Waters Viridis 2-EP and Phenomenex Luna HILIC) were used in tier 1 screen [2]. The compounds were initially analysed using a 10-40% modifier gradient (3 min). For easy separations, this method could directly be transferred to preparative scale, but for most compounds there was a need for further optimisation. Running several analyses for each compound was very time-consuming and therefore challenging samples were often purified using RPLC instead.

When working with RPLC, increased separation efficiency is achieved using focused gradients in preparative scale. The analytical retention time of the target compound in a generic gradient (2-94% ACN) is easily translated into a focused gradient (e.g. 10-60% ACN) used for preparative scale. Perhaps, this could also be done in SFC, if a reliable and robust scale-up methodology could be established. Separation of a few test compounds were performed and the initial result was promising *(one example given in Figure 1)*, so we decided to investigate this further.

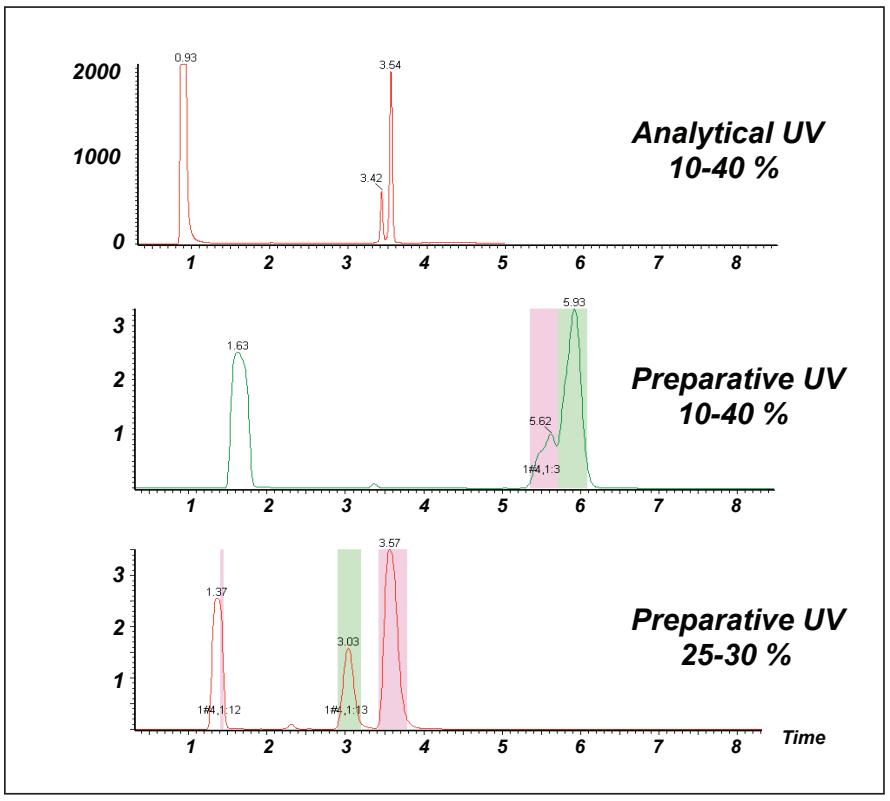

Figure 1. An example of scaling up in SFC from a generic gradient in analytical scale to a generic gradient versus a focused gradient in preparative scale.

The correlation between analytical retention times and the retention times in the preparative separation for different focused gradients was determined using a set of test compounds. The test set was composed of compounds with varying chemical properties, including neutrals, bases and acids [3]. It was decided that a shallow gradient, in which the percentage of modifier is increased by 5%, should be used as a starting point in the preparative scale. The correlation plots for this data are shown in *Figure 2*. The blue ellipse *(Figure 2a)* represents different compounds using the same focused gradient. The red ellipse *(Figure 2a)* shows the retention time in preparative scale for one single compound for which different focused gradients have been used [4,5].

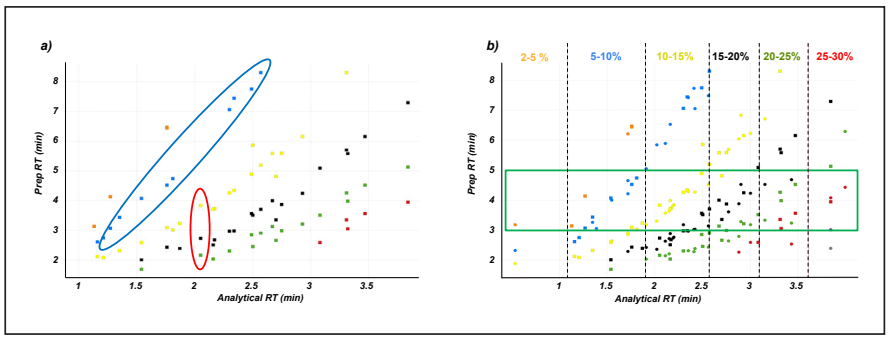

Figure 2. Correlation between retention times in analytical and preparative scale for the test compounds using different focused gradients. The markers are colored according to the focused gradient used in preparative scale.
a) Results using the Waters Viridis 2-EP column.
b) Results using the Waters Viridis 2-EP and the Phenomenex Luna HILIC column.

As all crude samples are dissolved in DMSO, this needs to be considered. DMSO is retained in SFC, and therefore it would be preferable to have a retention time of at least 2 minutes for the target compounds in the preparative run to avoid interference with the DMSO. The desired retention interval (3-5 min) is highlighted by the green box in *Figure 2b*.

Finally, six retention time intervals in analysis were selected which correspond to different focused gradients in preparative scale *(Figure 2b)*. The 2-5% gradient proved to be less successful due to a prolonged injection time as the sample is injected in the modifier flow. This results in peak broadening and the scale up becomes less predictable. Therefore, compounds with very low retention time in SFC are primarily purified using RPLC. The focused gradients were implemented in the purification process and later; the scale up methodology was evaluated by plotting the results for the crude samples in the same manner as for the test compounds *(Figure 3)*.

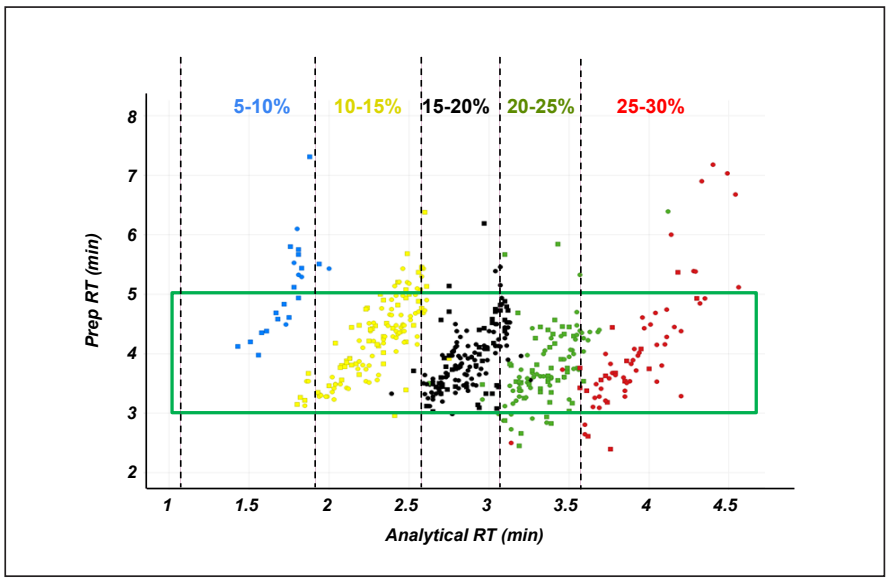

Figure 3. Correlation between analytical and preparative retention times for crude samples using different focused gradients. The green box represents the desired retention time window in preparative scale.

The overall performance was regarded as successful as the scale up became less time consuming and the focused gradients gave an improved separation for the target compound compared to running a generic 10-40% gradient. A higher percentage of the incoming samples were now purified using SFC, but the usage of SFC was still limited due to a lower sensitivity of the UV detector in the X5 system compared to the reversed phase UPLC system. In SFC analysis, it was often difficult to identify and quantify small impurities. The Waters X5 system was therefore replaced by a Waters Acquity UPC^2 system, which is far more robust and has a UV detector with higher sensitivity.

The Acquity UPC^2 system was compared with the Acquity UPLC system by analysing a mixture of test compounds. The quality of the results were similar and shows that peaks <1% can be detected equally well with the two systems *(Figure 4)*. When evaluating the initial results for preparative separations of the crude samples *(Figure 3)*, it was noticed that the scale up methodology was less successful for early and late eluting compounds. This could possibly be circumvented by expanding the analytical gradient and/or by introducing a larger number of overlapping gradients in the preparative scale.

The compounds used in the original test set for the scale up experiments were analysed on the UPC^2 system using columns with smaller particle size (3 or 3.5 µm) and shorter length (100 x 3 mm) and the generic gradient was expanded to 5-50% modifier (4 min).

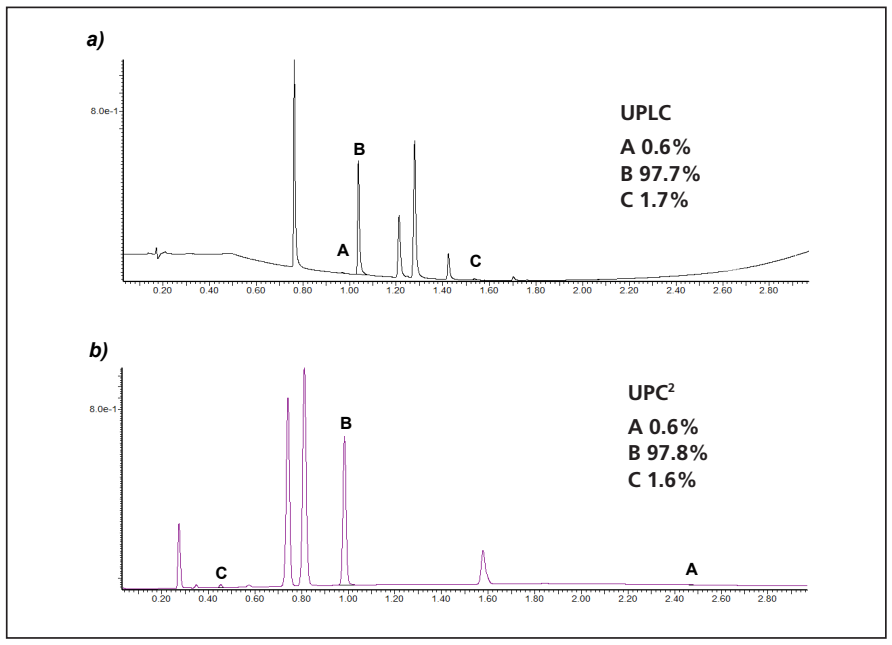

Figure 4. Analytical chromatograms for a mixture of compounds. The peaks corresponding to compounds A, B and C are integrated.
a) Waters Acquity UPLC system using a BEH C18 column (2.1 x 50mm, 1.7µm). Gradient elution 2-94% ACN (pH10, 0.2% NH_3) in 3 min, 1 ml/min, 45°C.
b) Waters Acquity UPC^2 system using a BEH column (3.0 x 100mm, 3.5 µm).Gradient elution 5-50% MeOH (20 mM NH_3) in 4 min, 2.5 ml/min, 40°C, 120 bar.

The analytical retention times were correlated to the known retention times in preparative scale and a larger set of overlapping focused gradients (e.g. 12-17%, 15-20% and 17-22%) was created using the same procedure as previously. The implementation and translation of gradients was fast and the UPC^2 system was used for real samples within one week after installation. The tier 1 screen was altered to include three columns (Phenomenex Luna HILIC, Waters BEH 2-EP and Waters BEH). The same scale up procedure is used for all columns and modifiers.

In our lab, the yield for target compounds during the purification process has been lower for the Waters Prep 100 SFC system compared to the Waters Autopurification HPLC MS system. When evaluating the yield of the SFC system, single fractions of the test compounds were collected. This is important as the collector probe makes a vertical movement when changing fraction tubes, during which the flow is diverted to waste. This means that the compound loss is even higher if several fractions are collected. The results showed that the loss was higher for small amounts. Initially, samples in scale <20 mg were therefore preferentially purified using RPLC.

In an attempt to determine where the major loss of compound occurs, recovery experiments were performed using different fraction collection settings. The alignment between the UV and MS detectors, the delay time between the MS detector and the fraction collector as well as different make up flows to the gas/liquid separator (GLS) unit was investigated. The results were evaluated by evaporating fractions overnight in tare weighed tubes followed by quantification by weight. The settings used in the purification process were shown to be optimal and minor adjustments did not influence the recovery.

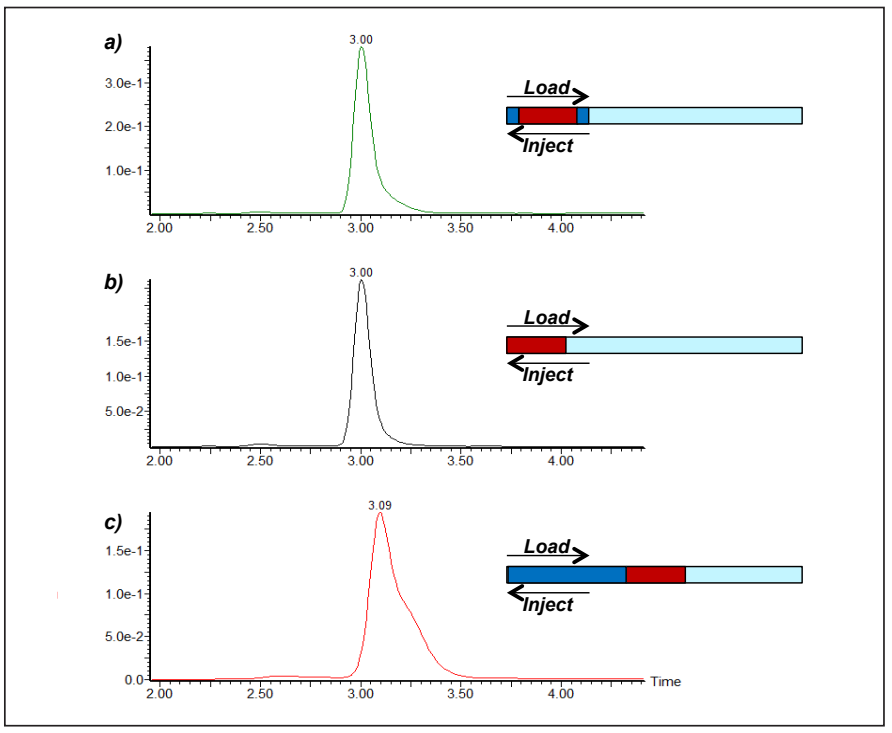

Figure 5. UV chromatograms for coumarin-314 using different injection techniques on the Waters Prep 100 SFC MS system. On the right is the schematic view of the loop for each injection technique. The sample volume is marked in red, pure methanol in dark blue and the modifier in light blue.
a) Sample sandwiching.
b) Partial injection.
c) Partial injection with center in loop.

The default setting for injection is partial loop on the Waters Prep 100 SFC system, but there are other possibilities for sample injection. These include partial injection centring in loop, sample sandwiching and full loop injection (not suitable for preparative work).

Standard recovery is routinely measured by injecting bensocaine (50 mg in 1000 µl MeOH) and the recovery was shown to increase from 90% using partial injection to 95% when using partial injection with the centre in loop option. This could indicate that there might be some sample solution left in the injection tubing after partial injection, but if the sample is centred in the loop using additional solvent, this loss is minimised. Centre in loop was also tested for smaller amounts (10 mg) and the general increase in recovery was 5-10%. This injection technique was evaluated for crude samples in the purification process. It was soon discovered that the peaks became broader due to solvent injection effects, caused by the relatively large addition of extra MeOH to centre the sample in the loop *(Figure 5)*.

The next attempt made was to use sample sandwiching which is a technique that we are already using for RPLC separations. Here small plugs of DMSO are used to prevent the sample precipitating during injection. In SFC, sample sandwiching using a small amount of MeOH (200 µl) could be used to rinse the tubing and ensure that all of the sample solution is pushed into the loop and injected onto the column. The standard recovery results were similar to those using centre in loop, i.e. recovery close to 95%. The experiment was repeated using just air gaps instead of MeOH but the recovery decreased (similar to that of partial injection without centre in loop). The results from the standard recovery tests using bensocaine (50 mg in 1000 µl) are summarised in Table 1. The results show that sandwiching and partial injection with centre in loop gives the highest recovery, but as centre in loop resulted in peak broadening, this technique was not further investigated.

To verify the result, the recovery experiment was expanded to include felodipine and 3-hydroxydiphenylamine. The compounds (10-50 mg in 200-1000 µl) were injected using isocratic conditions and sample sandwiching was found to be the superior injection technique *(Table 1)*. This was also true for smaller amounts. When using the focused gradients, predicted from the analytical runs on the UPC^2 system, the recovery was also higher when using the sandwiching technique.

Table1.

Compound	Amount (mg)	Volume (µl)	Method	Sandwiching (200µl MeOH)	Partial loop	Partial, center in loop	Air gaps
bensocaine	*50*	*1000*	*isocratic*	*95*	*89*	*95*	*91*
bensocaine	*25*	*500*	*isocratic*	*94*	*76*		
bensocaine	*10*	*200*	*isocratic*	*84*	*77*		
bensocaine	*50*	*1000*	*gradient (8-13%)*	*90**	*87*		
felodipine	*50*	*1000*	*isocratic*	*96*	*91*		
felodipine	*10*	*200*	*isocratic*	*88*	*82*		
felodipine	*50*	*1000*	*gradient (12-17%)*	*97*	*91*		
3-hydroxy diphenylamine	*50*	*1000*	*isocratic*	*97*	*90*		
3-hydroxy diphenylamine	*10*	*200*	*isocratic*	*91*	*81*		
3-hydroxy diphenylamine	*50*	*1000*	*gradient (20-25%)*	*100*	*92*		

**** The tail of the peak was not collected due to suboptimal fraction collection settings.***

For injection of very small amounts (e.g. 10 mg in 200 µl) the recovery is slightly lower, but there is still a significant difference between partial injection and sample sandwiching. An absolute value above 85% is regarded as acceptable for a purification system with these dimensions. Consequentially, the amount of sample is no longer a limitation for SFC. All crude samples purified using SFC are now injected using sample sandwiching with MeOH.

Conclusions

The time spent on analysis before purification has been decreased by replacing the Waters X5 SFC MS system with a Waters Acquity UPC^2 MS system. The quality of the chromatographic result in both UV and MS is now comparable to that from the Waters Acquity UPLC MS system. The UPC^2 system is also more robust than the X5 system.

Today, all samples are analysed using both SFC-MS and UPLC-MS. In SFC, the samples are analysed using a generic gradient (5-50% 20 mM NH_3 in MeOH) on three different stationary phases (Phenomenex Luna HILIC, Waters BEH and Waters BEH 2-EP). In UPLC, samples are analysed using a generic gradient (2-94% ACN) at pH 10 (0.2% NH_3, Waters BEH C18) and at pH 3 (10 mM HCOOH, Waters HSS C18). Here we have described how the analytical retention time for the most efficient separation is used to predict a focused gradient for preparative scale. The scale up methodology is only dependent on which chromatographic technique is used, i.e. SFC or RPLC, but it is independent of the column and mobile phase. This ensures a fast and generic scale up for all samples.

Initially, the recovery in SFC was lower than in RPLC especially for small amounts (<25 mg). This has been investigated by changing collection parameters and injection techniques. The recovery has been increased from 90% to 95% using sample sandwiching injection. Now, also small amounts are routinely purified using SFC.

The generic methods used in SFC are not applicable for all compound classes; most acids are still purified using RPLC. Investigations are now ongoing to find a more generic modifier that may be used for acids, bases and neutrals.

References

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