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Integration of supercritical fluid chromatography into drug discovery as a routine support tool Part I. Fast chiral screening and purification

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Abstract

Supercritical fluid chromatography (SFC) has been implemented within our group as a purity assessment and purification tool to complement high performance liquid chromatography (HPLC) for diastereomer and chiral separations. Using a novel strategy, rapid chiral screening has been implemented using short columns, high flow rates and fast gradients. A primary screen delivers a separation assessment using one solvent modifier (methanol) and four columns (Chiralpak AD-H and AS-H, and Chiralcel OD-H and OJ-H) run serially in a total of 24 min. A secondary screen then uses ethanol and isopropanol (IPA) modifiers across the same columns. The screens can be combined to run a sequence of samples overnight where each racemate is analysed over 80 min. The fast analytical screening and optimisation process enables rapid identification of the purification method. Furthermore, subsequent preparative chiral SFC has decreased the overall sample turnaround time for the Medicinal Chemist, delivering high fraction purities and acceptable recoveries, substantial operational cost savings and increased flexibility with respect to large scale purification feasibility in comparison to HPLC. SFC has been so successful it is now used as the primary method for chiral analysis and purification within our laboratory.

Keywords: Supercritical fluid chromatography; Enantiomer separation; Gradient screening; Chiral; Drug discovery

1. Introduction

Many molecular targets within drug discovery are chiral where individual enantiomers are required for biological testing. Rather than dedicating considerable time developing chiral synthetic routes, we have followed the strategy of separating the individual isomers using preparative high performance liquid chromatography (HPLC). The utilisation of HPLC for chiral separation is well documented [1–5]. During recent years we have developed a normal-phase chiral HPLC gradient screening strategy [6–11] based on solvent and column switching as reported by Whatley [12] and Villeneuve and Anderegg [13]. Our current HPLC screen can run a racemate across 4 columns and 2 solvent systems in 3 h. Although it is an ongoing requirement to increase throughput and capacity, we believe it is not currently possible to reduce the 10 min HPLC gradient run times without compromising separation. Recently, our group implemented supercritical fluid chromatography (SFC). The intention was to utilize the speed, solvent handling and cost benefits of SFC as well as provide a new separation tool for specialised separation method development and purification.

SFC is a well established separation technique [14–18]. The low viscosity and high diffusivity of the SFC mobile phase allow for separations to be achieved with higher flow rates and longer columns. Compared to normal-phase HPLC, SFC offers higher efficiencies, improved resolution and faster column re-equilibration resulting in much faster separations. Packed SFC columns typically provide a three to five fold reduction in analysis time over HPLC [19–20]. SFC, sub-critical fluid chromatography, unified chromatography and

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enhanced fluidity chromatography are commonly used terms to describe the use of mobile phases operated near or above the critical parameters. Typically CO_2 is used as the supercritical fluid. As a polar modifier, such as methanol, is required to elute polar analytes, most SFC applications are operating under subcritical conditions. In our laboratory, it is accepted we may be operating under both supercritical and subcritical conditions across any given gradient but as this has not impacted separations, we are not concerned if the fluid is supercritical or subcritical. Therefore, we will use SFC as the single descriptor from hereon.

The application of SFC to chiral analysis is also well documented [20–27]. Recently, there have been a number of reports on the utilisation of SFC for rapid chiral screening [13,28–31]. During 2003 our group implemented SFC as a purity assessment and purification tool to complement HPLC for isomer and chiral separations. Building on the knowledge gained from our HPLC screen, we implemented 2.5 min gradient methods for chiral SFC screening. Therefore, SFC can screen a sample through 4 columns (AD-H, OD-H, OJ-H and AS-H) and 3 solvent systems (CO_2/MeOH, CO_2/EtOH and CO_2/IPA) in 80 min using a flow rate of 5 ml/min. Currently the overall screen time includes the injection of diluent solvent blanks following the switch to each new column: this is optional and their removal further reduces the screen time to 50 min, compared to 3 h by HPLC.

The fast chiral screen is used for rapid identification of the most appropriate column and modifier before scaling up to preparative SFC. Following column and modifier selection, all compounds are then run isocratically to develop the conditions most appropriate for purification. Compounds that have shown very poor resolution on the gradient screen, but considered 'hits', have usually been separated to baseline resolution when moving from gradient to isocratic modes through simple adjustment of the isocratic modifier percentage.

Preparative SFC [16,18,20,32] offers the same benefits as analytical in addition to reduced solvent handling, reduced operational costs and most significantly, reduced fraction volumes in a readily evaporated solvent. Preparative chiral SFC examples are discussed, illustrating these benefits.

Initially, both analytical HPLC and SFC were run in parallel to select the most appropriate technique for purification. During the last 12 months, 96% of chiral purifications were selected for SFC ahead of HPLC. During this period 64% were purified using the AD-H column, 10% using the OD-H column, 17% using the OJ-H column and 9% using the AS-H column. In addition, due to the solvent handling benefits of SFC, the group can support up to 60 g purifications compared to the previous 5 g maximum threshold supported by HPLC using the 21 mm i.d. columns, since we have no facility to handle large volumes of waste HPLC solvent. The parallel screening across both HPLC and SFC resulted in delays due to the necessary lag time while the slower HPLC analysis was completed. Therefore, we have decided upon SFC as the primary default method for chiral analytical method development and purification, utilising HPLC as a secondary technique for samples failing to separate by SFC.

2. Experimental

2.1. Materials and reagents

Chiral methods were optimised using sets of commercially available standard compounds alongside compounds submitted into the group as part of the routine support service to Discovery Chemistry. *trans*-Stilbene oxide and propranolol were used for chiral SFC column performance checks supplied by Sigma-Aldrich (Gillingham, UK). Carbon dioxide (99.95% purity) was obtained from BOC Gases (Worsley, Manchester, UK). Heptane, methanol, ethanol and isopropanol were HPLC grade from Fisher Scientific (Loughborough, UK).

2.2. Additives

Isopropylamine (IPAm), diethylamine (DEA), dimethylethylamine (DMEA), triethylamine (TEA) and trifluroacteic acid (TFA) have been used as additives to improve peak shape and separation, available from Sigma-Aldrich. IPAm is the preferred additive as it delivers the sharpest peaks for strongly basic compounds. On occasion, IPAm has proved difficult to remove from fractions due, we believe, to the formation of the carbamate from reaction with CO_2 [33,34]. Although a combination of centrifugal evaporation under reduced pressure and freeze-drying removes the additive from these samples, we continue to look for a suitable tertiary amine replacement. The majority of compounds analysed are basic. Acidic compounds are analysed separately using TFA as the additive.

2.3. Stationary phases

Columns packed with Chiralpak AD and Chiralpak AS (amylose derivatives) and Chiralcel OD and OJ (cellulose derivatives) were used for both chiral HPLC and chiral SFC experiments, purchased from Chiral Technologies (Chiral Technologies Europe, Illkirch, France). Column dimensions were 15 cm × 4.6 mm i.d., 5 μm particle size for HPLC gradient screening and 10 cm × 4.6 mm i.d., 5 μm particle size for SFC gradient screening. Some preliminary SFC experiments were performed using 25 cm × 4.6 mm i.d., 10 μm particle size. For preparative SFC the column dimensions were either 25 cm × 20 mm i.d., 10 μm particle size or 25 cm × 21 mm i.d., 5 μm particle size.

2.4. Instrumentation

SFC analysis was performed on a Berger Minigram system configured with 6-way column and solvent switching. SFC purification was performed on a Berger Multigram II system. Both systems were equipped with a Knauer variable wavelength UV detector, supplied by Mettler-Toledo AutoChem

(Leicester, UK). Liquid CO_2 is delivered a distance of 210 m to the laboratory by a Berger GDS-3000 system supplied also by Mettler-Toledo AutoChem. Twin pairs of 200 l capacity dewars are ganged together to deliver a continuous supply of CO_2. HPLC analysis was performed on a Waters 2695 Alliance system configured with six-way column and quaternary solvent delivery supplied by Waters (Hertfordshire, UK).

2.5. Chromatographic conditions

The experimental conditions for each generic gradient method are described in Table 1. The 6 min SFC gradient method using the 25 cm length columns was used for a brief implementation period following the installation of the SFC equipment. Blank injections were performed following the switch to each new column in the screen. This is not a requirement but was considered beneficial to understand the background profile for each column and solvent combination. The column equilibration time following a switch to a new column was 1.0 min and the modifier equilibration time following a switch to a new solvent was 5.0 min. The overall instrument time is 24 min for the primary screen, 52 min for the secondary screen and 80 min if both screens are combined in a single run. By removing the blank injections a reduced screen time of 14 min, 32 min and 50 min, respectively, is possible. Isocratic methods for preparative SFC were developed for each sample in turn following the initial gradient screen and are mentioned in the context of results and discussion. Scaling from analytical to preparative conditions was an indirect process. The analytical isocratic result on a 10 cm × 4.6 mm i.d. column using a flow rate of 5 ml/min was scaled up to a 25 cm × 21 mm i.d. column using an appropriate flow rate for the separation varying between 50 and 70 ml/min. Samples for purification were dissolved in the running modifier.

2.6. SFC column performance checks

Analytical chiral columns are performance checked with *trans*-stilbene oxide using the chiral gradient screen. Regular checks are not necessary due to the extended lifetime of columns using SFC. The first set of chiral columns has been used over a period of 18 months where 7665 injections were made, with no loss in performance. Preparative chiral columns are also checked on a case by case basis using either *trans*-stilbene oxide or propranolol.

3. Results and discussion

3.1. Analytical SFC speed benefit compared to HPLC

Using SFC, compounds can be screened and separations optimised much faster than HPLC. The majority of separations are complete within 4 min, compared to the 20 min usually encountered with HPLC. As an example, Fig. 1

Table 1
Experimental conditions for the two analytical chiral SFC gradients and the analytical chiral HPLC gradient

Technique	Gradient conditions						Run time (inj to inj)	Solvents	Temperature (°C)	Outlet pressure (bar)	Flow rate (ml/min)	Column stationary phase	Column dimensions and particle size
	Rate	Modifier (%)	Hold (min)	Time	%A	%B							
SFC chiral analytical	–	5	0.5	–	–	–	6.0	MeOH + 0.4% DEA, EtOH + 0.4% DEA, IPA + 0.4% DEA as modifier	35.0	100.0	5.0	Chiralpak AD and AS, Chiralcel OD and OJ	25 cm × 4.6 mm, 10 μm
	15.0	50.0	1.0	–	–	–							
	99.0	5.0	1.0	–	–	–							
SFC chiral analytical	–	10.0	0.0	–	–	–	2.5	MeOH + 0.2% IPAm, EtOH + 0.2% IPAm, IPA + 0.2% IPAm as modifier	40.0	120.0	5.0	Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H	10 cm × 4.6 mm, 5 μm
	23.5	55.0	0.59	–	–	–							
HPLC chiral analytical	–	–	–	0.0	90	10	10.0	A = Heptane + 0.2% IPAm B = EtOH or IPA + 0.2% IPAm	40.0	–	1.0	Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H	15 cm × 4.6 mm, 5 μm
	–	–	–	1.0	90	10							
	–	–	–	5.0	40	60							
	–	–	–	7.0	40	60							
	–	–	–	7.25	90	10							
	–	–	–	10.0	90	10							

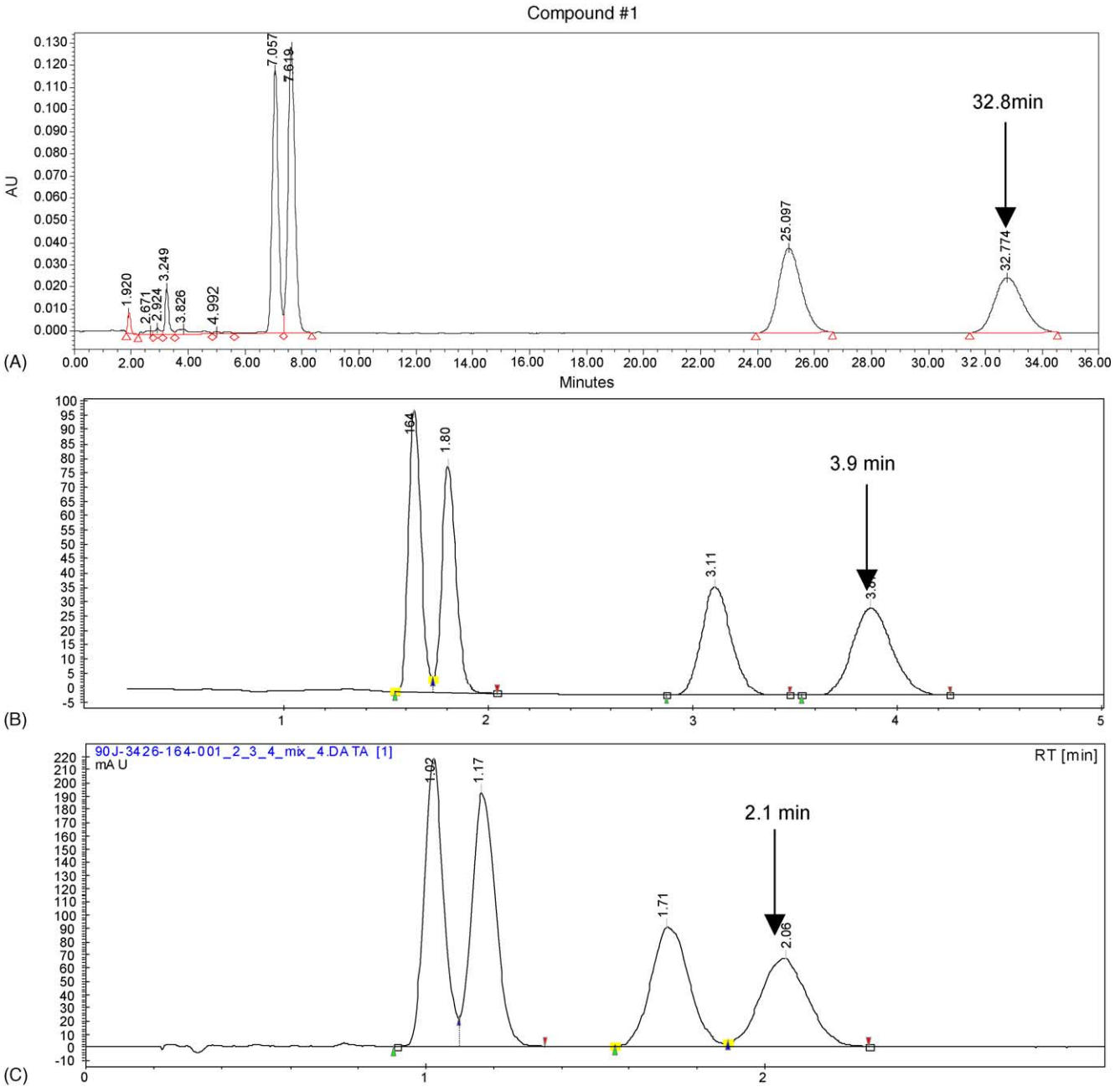

Fig. 1. Compound **1** containing two chiral centres analysed isocratically by HPLC (A) using 85% Heptane, 15% Ethanol each with 0.2% DEA on the chiral AD-H 150 mm × 4.6 mm column, flow rate 1 ml/min; SFC (B) using a modifier of 15% MeOH containing 0.4% DEA on the AD 250 mm × 4.6 mm column, flow rate 5 ml/min; SFC (C) using a modifier of 12% MeOH containing 0.2% IPAm on the AD-H 100 mm × 4 mm column, flow rate 5 ml/min. SFC outlet pressure was set to 100 bar and temperature was 35 °C. Detection by UV, 260 nm for HPLC and 220 nm for SFC.

shows the chiral separation of all four isomers from a diastereomeric mixture. Separation of all four isomers was achieved using HPLC on a single chiral column of dimensions 150 mm × 4.6 mm (AD) and a flow rate of 1 ml/min. We considered the resolution of all four isomers within 35 min acceptable and a success. Directly following the SFC installation the same mixture was analysed by SFC where all four isomers were successfully separated within 5 min using a flow rate of 5 ml/min and the 25 cm × 4.6 mm AD column. With the current column dimensions of 10 cm × 4.6 mm (AD-H) and a flow rate of 5 ml/min the run time was reduced further to just over 2 min. The SFC modifier composition was changed from 15 to 12% to maintain approximately the same resolution between the first two eluting enantiomers. For this particular example SFC is a factor of 15.6 faster than HPLC.

3.2. Analytical chiral screen

An example of a primary screen is presented in Fig. 2. Although separation is observed on both the AD-H and OD-H

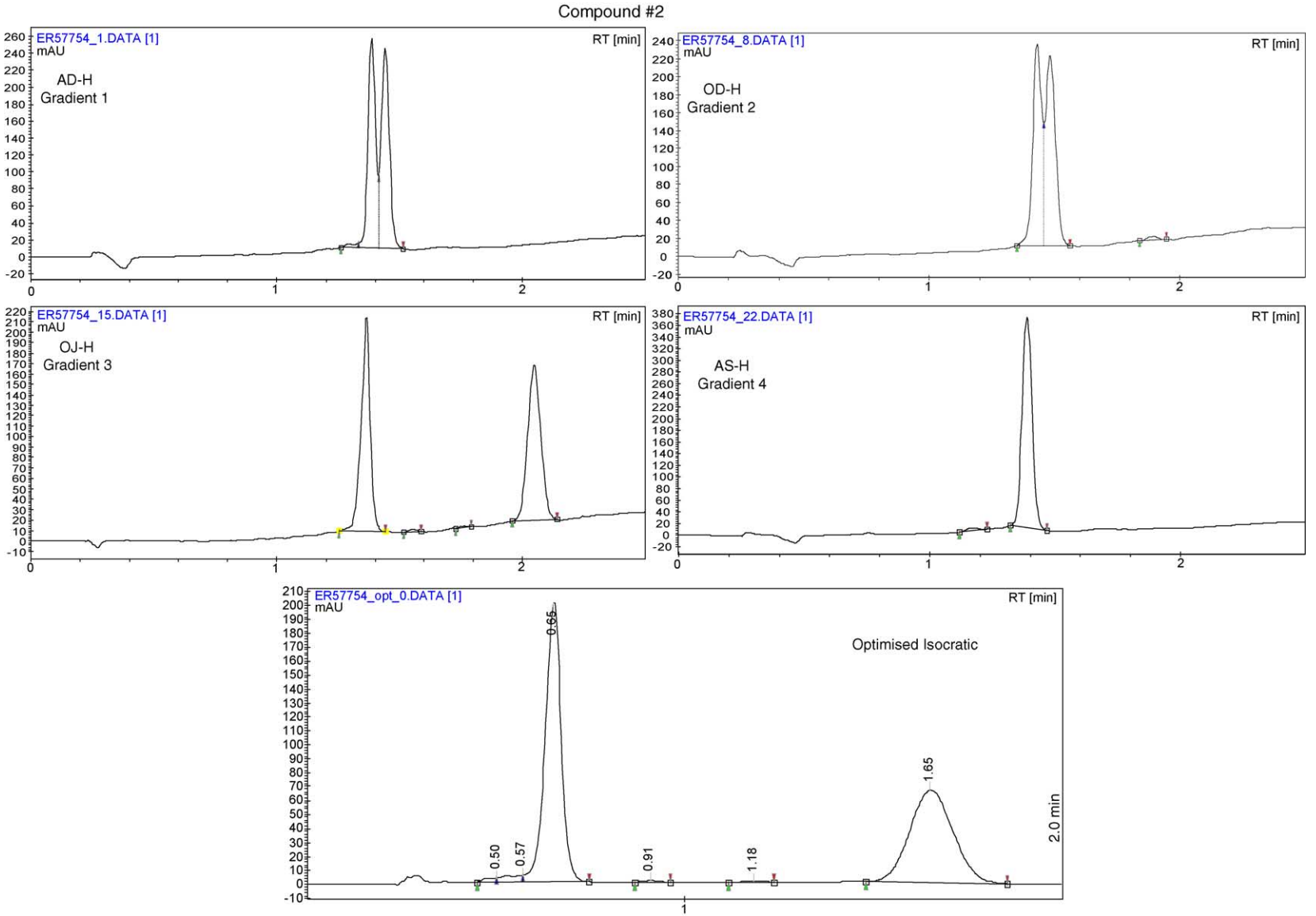

Fig. 2. Compound **2** analysed using the primary chiral screen identifying the OJ-H column as the column of choice for purification. The optimised isocratic conditions were 30% MeOH containing 0.2% IPAm on the OJ-H column, flow rate 5 ml/min. Outlet pressure was set to 120 bar and temperature was 40 °C. Detection by UV at 220 nm.

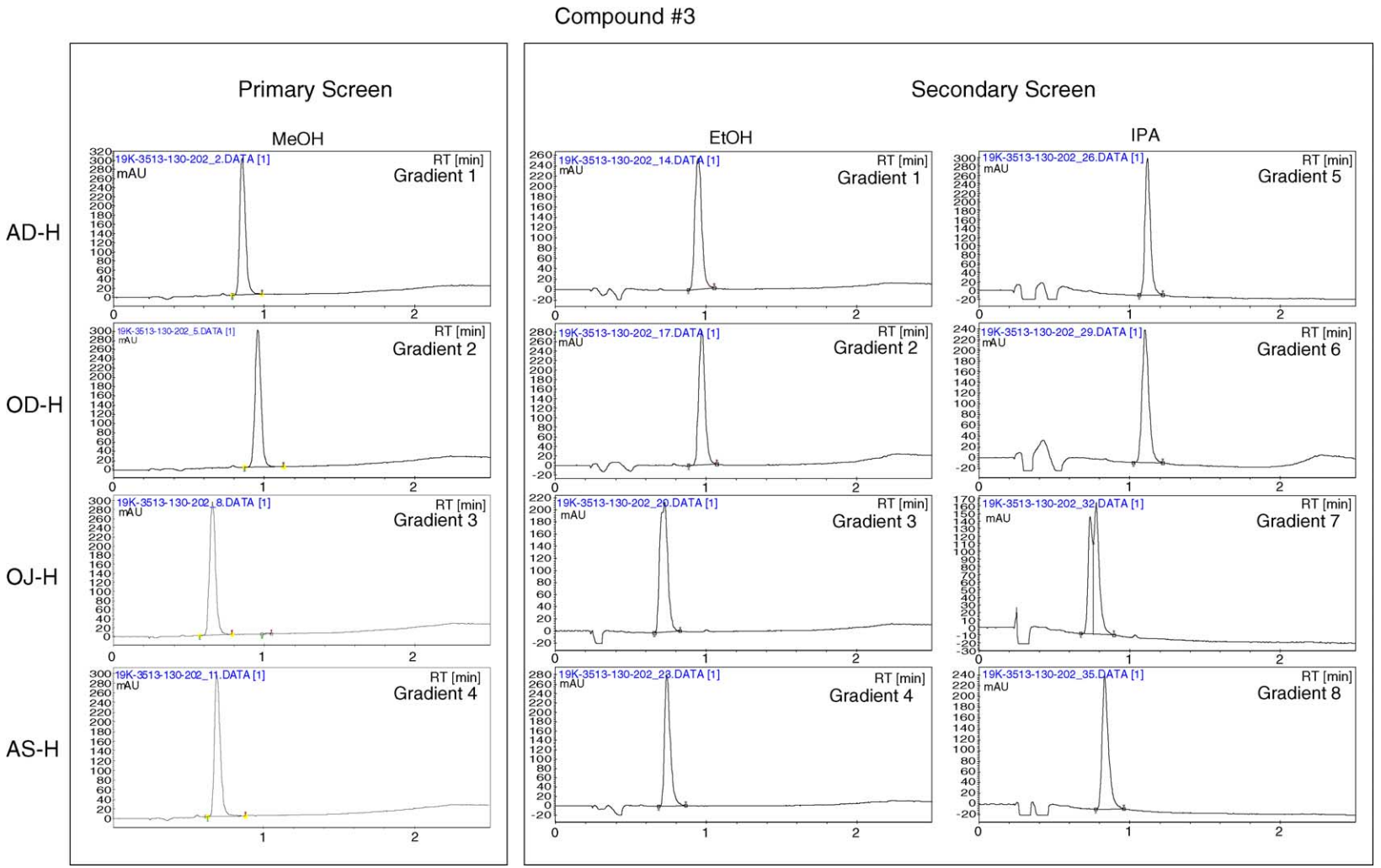

Fig. 3. Compound **3** analysed using first the primary chiral screen and subsequently the secondary screen, identifying the OJ-H and IPA (with 0.2% IPAm) as the column and modifier combination of choice.

columns, a far superior separation is achieved using the OJ-H column. This column is the obvious choice for preparative SFC as the facile separation allows for large column loadings and will potentially deliver a higher productivity compared with the AD-H and OD-H. The isocratic method optimisation process is fast due to the short run times, usually 3–4 min, and fast equilibration times. Identification of the isocratic conditions following the gradient screen is a judgement call by the analyst based on gradient retention. This particular example elutes late in the gradient so a high percentage of modifier was selected for the isocratic method. During a 12-month period 48% of chiral purification conditions were optimised from the primary screen alone where the overall selection and optimisation of the preparative SFC method is easily achieved in less than an hour.

Fig. 3 is an example where the racemate did not separate using the primary screen but the subsequent secondary screen partially resolved the enantiomers on the OJ-H column using IPA with 0.2% IPAm as the modifier. Although only partial separation was achieved using the gradient, baseline resolution was obtained when the compound was run isocratically. Fig. 4 presents the final purity assessment of each collected enantiomer, using the optimised isocratic conditions of 7% IPA with 0.2% IPAm, following chiral purification. The challenging separation allowed for only 10 mg loadings but this was sufficient to purify the required 100 mg in just over an hour.

Multiple samples and acidic compounds are occasions when both primary and secondary screens are combined. Fig. 5 is an example of a combined screen set up for a compound containing acidic functionality. The material was known to be a mixture of diastereomers, in the approximate ratio of 60:40 from NMR experiments and a separation between all four enantiomers was required. The IPA modifier and the AD-H column was the only combination that gave separation between all four isomers. The isocratic method of 25% IPA with 0.1% TFA was developed delivering base line resolution between all isomers in under 5 min. The integration results of 19% (peak 1), 31% (peak 2), 19% (peak 3) and 30% (peak 4) can be used to assign the individual diastereomeric pairs of enantiomers to peaks 1 and 3, and peaks 2 and 4.

The transfer from isocratic analytical separation to preparative SFC is performed indirectly such that separation results performed on 10 cm length columns at 5 ml/min are transferred to 25 cm length preparative columns using flow rates of between 50 and 70 ml/min. In our experience analytical separations reproduce very well when samples are scaled up to the preparative system. In fact enhanced separation is expected and usually observed due to the additional preparative column length.

3.3. Purification

The speed benefit of SFC achieved for analytical separations also applies to preparative separations where separations tend to be approximately five times faster than achieved by HPLC. We have found the average system productivity to be 1.0 g h^{-1} but where separation and solubility have not presented an issue, we have purified samples up to a productivity rate of 8.5 g h^{-1} using a conventional 21.2 mm diameter column and a flow rate of 70 ml/min. An example of a chiral purification is presented in Fig. 6. The analytical screen identified the presence of an impurity eluting on the tail of enantiomer 1 (e 1). Fractions were, therefore, collected by time, not threshold, to enrich the final purity of the first eluting enantiomer. A total of 22 stacked injections were performed where peaks eluted exactly at the predicted

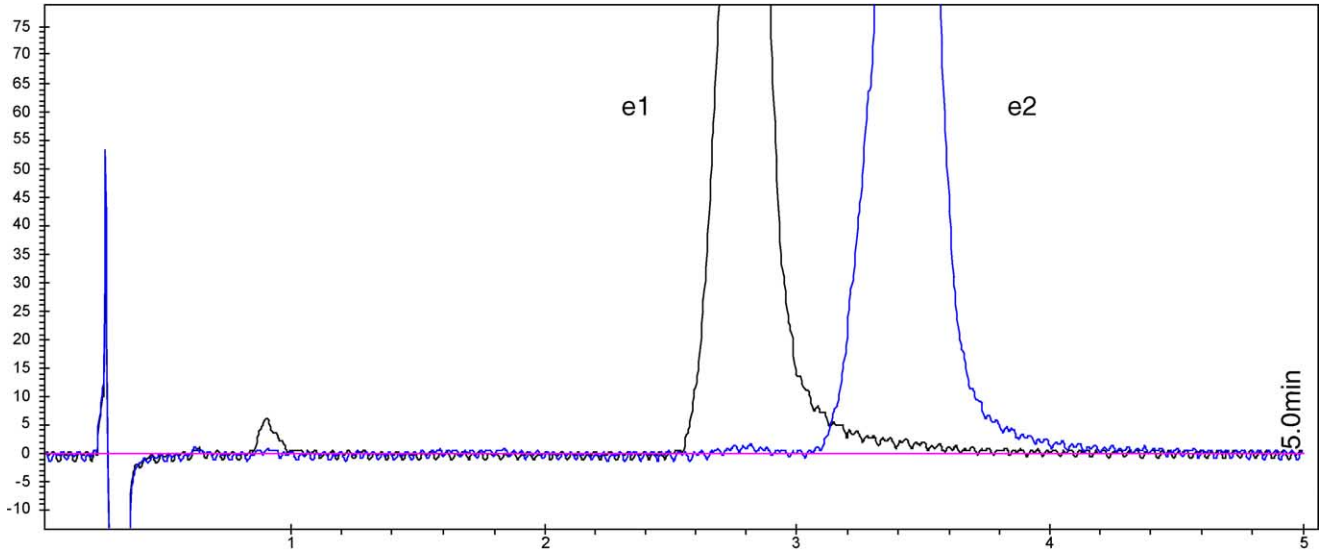

Fig. 4. Fraction purity assessment following the purification of compound **3** represented in Fig. 3. Optimised isocratic conditions were 7% IPA containing 0.2% IPAm on the OJ-H column, flow rate 5 ml/min. Outlet pressure was set to 100 bar and temperature was 35 °C. Detection by UV at 220 nm.

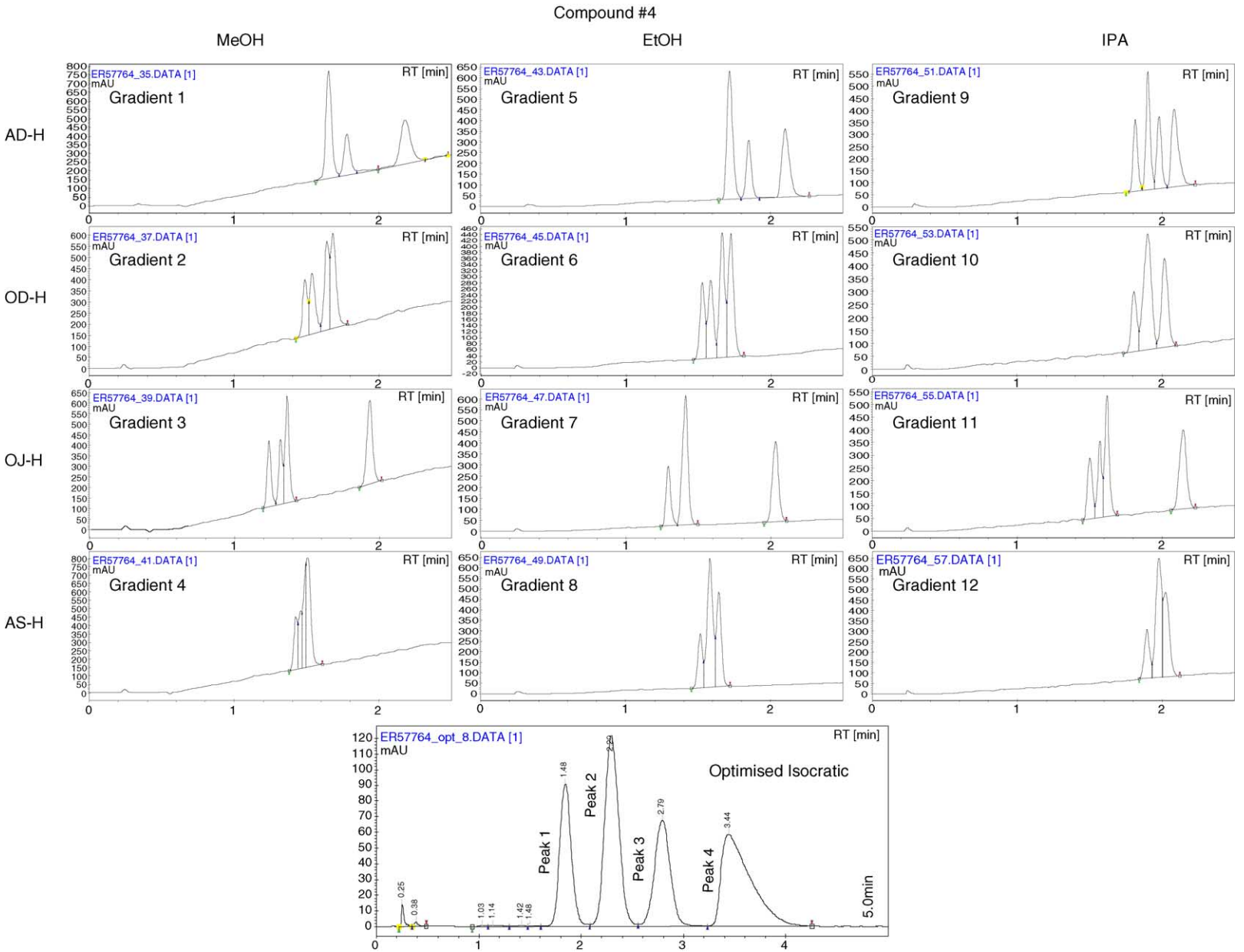

Fig. 5. Compound **4** analysed using the combined primary and secondary chiral screens identifying the AD-H and IPA (with 0.1% TFA) as the column and modifier combination of choice. The optimised isocratic conditions were 25% IPA containing 0.1% TFA on the AD-H column, flow rate 5 ml/min. Outlet pressure was set to 120 bar and temperature was 40 °C. Detection by UV at 220 nm.

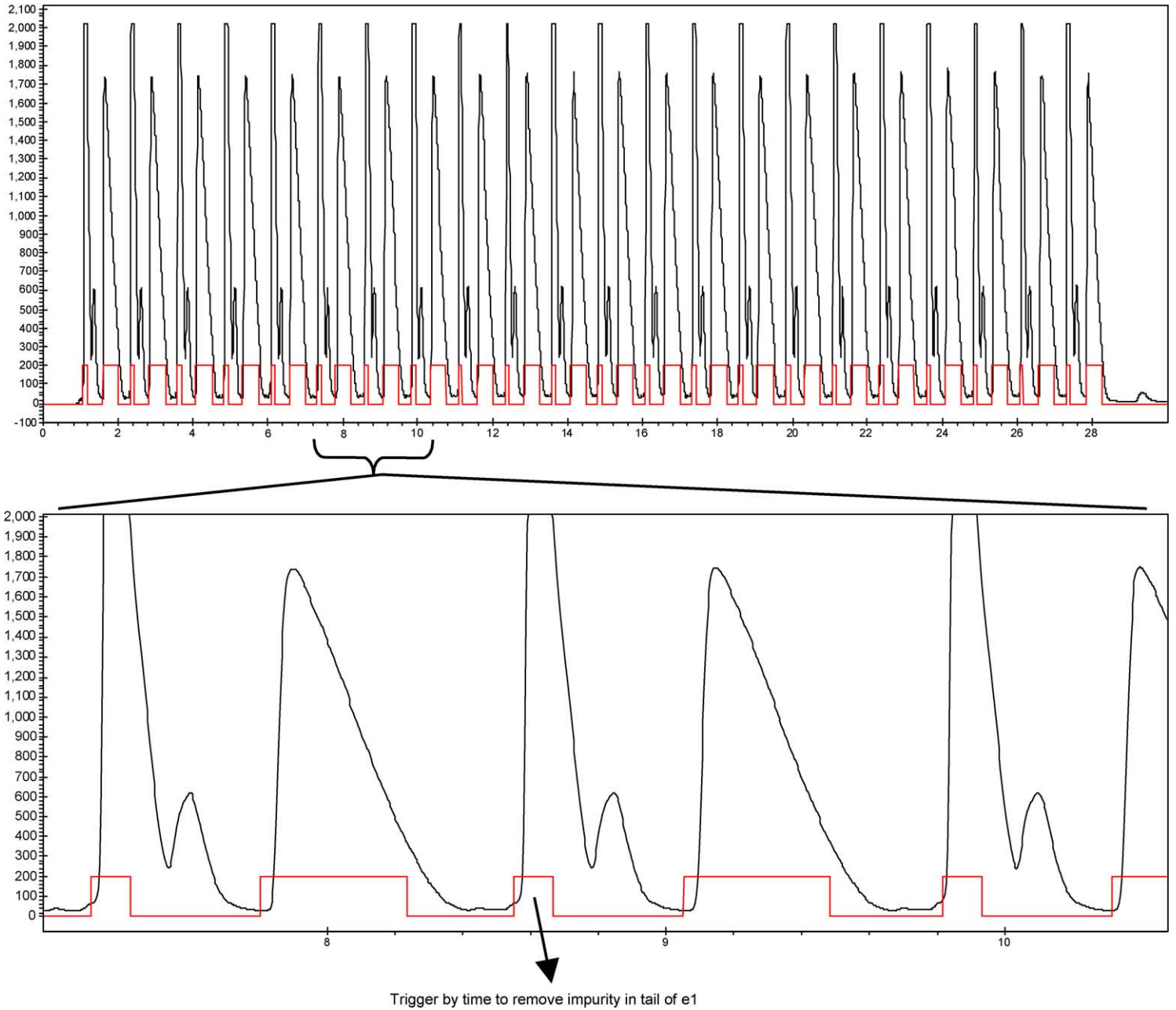

Fig. 6. Preparative chiral SFC example collecting by time windows. Twenty-two stacked injections of 72 mg are displayed. The flow rate was 70 ml/min using the AD (10 μm) with dimensions of 25 cm × 20.0 mm diameter.

time resulting in the collection of clean enantiomers. Since the system automatically collects between fractions, a third fraction was isolated between enantiomer 1 (e 1) and enantiomer 2 (e 2) that was enriched in e 1 and impurities. This purification application illustrates the impressive injection to injection reproducibility of SFC where retention time of the target components is very predictable, a critical requirement for stacked injections to work correctly. Purifications are regularly run overnight, where multiple stacked injections are performed without any hardware or software failure.

Overall system recovery was evaluated by running a 1.5 g purification at 50 ml/min requiring a very low modifier percentage of 3%. Eighty stacked injections were performed delivering approximately a 5 ml fraction volume for each enantiomer. An overall weight recovery of 91% was achieved. On one occasion, solid material was collected as the compound precipitated on collection without any loss in recovery. Over a 12-month period, the average final fraction purity was 97.9% and the average final weight recovery was 84.1%. A total of 209.7 g of material was purified.

Health and safety regulations restrict us from supporting HPLC purifications of >5 g due to the volumes of solvent required. These separations are generally run at other laboratories within the company equipped to handle larger volumes of solvent. This is no longer a concern when using SFC due to the continuous supply of CO_2 and the significant reduction in fraction volume. Our group can now support 60 g purifications, assuming adequate separation and sample solubility can be achieved.

Over a typical 12-month period approximately 3800 l of CO_2 was used. This represents a saving of approximately £40,000 in operational expenses, or a saving of £200 for each sample purified, assuming an identical consumption of HPLC grade Heptane if HPLC had been the method of choice.

4. Conclusion

The proposed SFC chiral gradient screening strategy delivers fast method development for the separation of enantiomers before their transfer to preparative SFC. To our knowledge, this is the first report on the use of short chiral columns and fast gradients for rapid chiral screening. Separations developed on the 10 cm length Daicel columns are successfully scaled up to the 25 cm length × 21.2 mm diameter columns without the requirement for conventional direct scale up experiments. SFC for purification has been demonstrated to deliver higher productivity, reduced operational costs and a health and safety compliant process for large-scale (∼50 g) purifications. In our laboratory SFC has replaced HPLC for chiral analysis and purification as the primary technique of choice.

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