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Comparison of preparative reversed phase liquid chromatography and countercurrent chromatography for the kilogram scale purification of crude spinetoram insecticide

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ABSTRACT

Reversed phase HPLC (RP-HPLC) and high performance countercurrent chromatography (HPCCC) were compared for the pilot scale purification of two semi-synthetic spinosyns, spinetoram-J and spinetoram-L, the major components of the commercial insecticide spinetoram. Two, independently performed, 1 kg, purification campaigns were compared. Each method resulted in the isolation of both components at a purity of >97% and yields for spinetoram-J and spinetoram-L of >93% and ≥63% of theoretical, respectively. The HPCCC process produced a 2-fold higher throughput and consumed approximately 70% less solvent than preparative scale RP-HPLC, the volume of product containing fractions from HPCCC amounted to 7% of that produced by HPLC and so required much less post-run processing.

1. Introduction

The spinosyns are members of a new class of polyketide-derived macrolides which are effective against a broad range of insect pests [1]. Spinetoram, a mixture of spinetoram-J and spinetoram-L, is prepared by the chemical modification of spinosyn J and spinosyn L which are fermentation-derived, natural products produced by the actinomycete, *Saccharopolyspora spinosa*. Although other polyketides have been discovered and used as insect control agents, the spinosyns have a unique structure, spectrum of activity and mode of action. As derivatives of naturally occurring compounds, these insecticides pose less environmental risk than many totally synthetic pesticides.

Structurally, spinetoram-L is distinguished from spinetoram-J by the presence of a double bond and a methyl group at the C-6 position (Fig. 1).

Periodic production of analytical standards of spinetoram-J and spinetoram-L from 1 kg batches of spinetoram has been traditionally accomplished using preparative RP-HPLC. In an effort to reduce the cost of this process, high performance countercurrent chromatography (HPCCC) was investigated. Herein, the RP-HPLC and the HPCCC processes used in the isolation of these two principal components are described and compared.

HPCCC is a chromatographic method which is complementary and orthogonal to HPLC [2]. It is a novel variant of countercurrent chromatography (CCC) which allows the use of high centrifugal force fields ($240 \times g$) resulting in better liquid stationary phase retention which consequently permits the use of higher flow rates and hence shorter run times and improved throughput (mass processed/time) compared with other forms of CCC whilst maintaining the technique's benefits which are demonstrated by the data presented and are outlined below [3]. The HPCCC column takes directly the role of the packed-bed column in an HPLC system.

Chromatographic resolution is the arithmetic product of retention, selectivity and efficiency factors. HPCCC is a low efficiency method and resolution depends mainly upon the control of highly tuneable selectivity [4].

The technique offers the preparative scale chromatographer several advantages: the active stationary phase volume is typically 70–90% of the HPCCC column volume and produces very high dynamic loading capacity which has a significant impact on chromatographic throughput (mass of solute processed per unit time)

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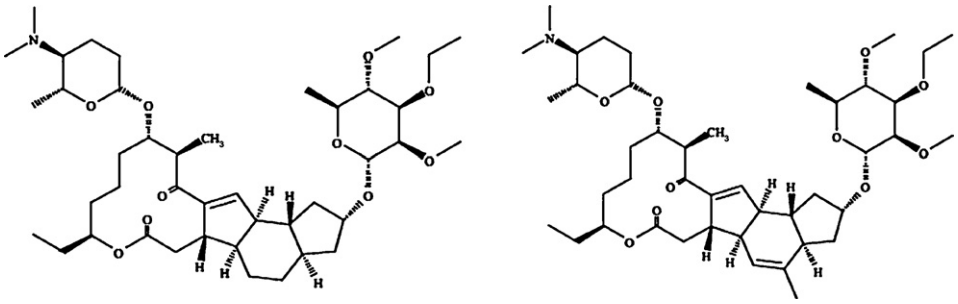

Fig. 1. Spinetoram: structures of spinetoram-J and spinetoram-L.

and high solute concentration in fractions collected which means easier downstream processing; HPCCC exhibits no non-primary solute–solvent interactions, i.e. there are no interferences from interactions between solutes and the liquid stationary phase; both stationary and mobile phases are liquids and either phase can take the role of stationary or mobile phase. The consequences of having a cheap, non-destructible stationary phase are obvious and mean that a new column is readily prepared as and when required; stacked injection methodologies are possible and other elution modes, such as elution–extrusion which considerably reduces run times and further decreases solvent consumption, are possible when using HPCCC; the technique is generally tolerant of contaminants such as unspent reagents/reactants or heavy metals and their ions either in solution or as suspended solids; finally since there is no solid, stationary phase present in an HPCCC system, there is no possibility of irreversible binding or chemical interaction with the stationary phase and recoveries are high: often essentially quantitative.

Properly designed and constructed HPCCC columns allow direct, linear, volumetric scale-up which is facile compared with other forms of modern CCC instrumentation [5].

In 1949 when Craig invented an efficient countercurrent distribution device (CCD), a single experiment could take days and the normal method development process of running trial CCC experiments could take an extraordinarily long time. Now that HPCCC experiments have durations similar to those of HPLC, method development by experimental solvent system scanning is feasible and usually takes no more than a few hours and can be much less if automated, 'on demand' mixing is employed. Potentially, any combination of reasonably volatile solvents that forms two immiscible phases, most usefully in more or less equal volume proportions and in which the phases separate in a reasonably short time, is useable for the performance of an HPCCC experiment. However, it is generally easier and more convenient to screen selected members of well characterised and widely used solvent system series such as the original HEMWat [6], and Arizona [7] systems which are examples of such series and comprise stepped polarity series of mixtures of hexane, ethyl acetate, methanol and water in fixed relative volume proportions for each polarity step.

Very few large scale separations involving more than tens of grams of sample, using countercurrent chromatography have been reported. The largest scale separation reported in the literature to date is the preparation of 1 kg of the glucosinolate natural product glucoraphanin using a 4.6 L HPCCC instrument [8,9]. The same HPCCC instrument was used to prepare 50 g of the monoterpene natural product honokiol [10]. A 5 L fast centrifugal partition chromatograph (FCPC) was used to process 22 g of a chloroform extract of *Xanthium macrocarpum* for the isolation of three sesquiterpene lactones [11]. The latter study compared FCPC with silica gel chromatography with respect to isolate purity and recovery. The scarcity of such separations is no doubt due to the fact that such large scale, reliable instruments have only become commercially available in the last few years. Additionally, since they provide considerable production capacity, many separations performed on these instruments are no doubt of a confidential nature.

2. Experimental

2.1. Solvents

All solvents used for analytical and preparative scale RP-HPLC and HPCCC were of HPLC grade.

2.2. Materials

Spinetoram (batch number WC1743CD023) was provided by Dow AgroSciences LLC. This sample contained a ratio of approximately 3:1 of spinetoram-J/L with an estimated purity of 90–92%. Other components within the sample were structurally related minor factors from the fermentation.

2.3. Equipment

2.3.1. Dow AgroSciences LLC (Indianapolis, Indiana, USA)

2.3.1.1. Analytical RP-HPLC. Analytical HPLC was performed on a Waters 600E solvent delivery module equipped with a 996 photodiode array detector, a 717 autosampler, and Empower workstation software (Waters Inc, Milford, MA, USA). The analyses were performed on a Gemini™, 4.6 mm × 250 mm, 5 μm, C18 analytical column (Phenomenex Inc, Torrance, CA, USA).

2.3.1.2. Preparative RP-HPLC. For preparative HPLC, a Varian PrepStar™, SD-2 solvent delivery module with a 530 fluidics module, a 325 dual wavelength detector, a Prostar™, 210 solvent delivery module (sample injection pump), and LC Responder™ workstation (Varian, Palo Alto, CA, USA) were used.

The column used was a Prochrom™, LC110 dynamic axial compression, I.D. 11 cm (NovaSep, Pompey, France). The column was packed with approximately 1200 g of Xterra, C8, 10 μm (Waters Inc, Milford, MA, USA) to produce a bed height of approximately 25 cm yielding a bed volume of 2375 mL and void volume of 800 mL.

2.3.2. Dynamic extractions Ltd (Slough, UK)

2.3.2.1. Analytical RP-HPLC. Analytical RP-HPLC was performed on a Varian Prostar 210 solvent delivery module equipped with a Varian Prostar 325 LC single wavelength detector, a Varian Prostar Model 430 autosampler, and Galaxie workstation software (Varian Ltd, Oxford, UK,). The analyses were performed on a Gemini™, 4.6 mm × 150 mm, 5 μm, C18 analytical column (Phenomenex Ltd, Macclesfield, UK).

2.3.2.2. HPCCC. Analytical and semi-preparative scale experiments were performed on a Dynamic Extractions Spectrum™ instrument (Slough, UK) which was fitted with an analytical scale column with

a volume of 22 mL, an I.D. of 0.8 mm, a β-value range from 0.64 to 0.81, and a revolution radius of 85 mm and a semi-preparative scale column with a volume of 132 mL, an I.D. of 1.6 mm a β-value range of 0.52–0.86, and a revolution radius of 85 mm.

Preparative scale experiments were performed on a Dynamic Extractions Midi™ instrument (Slough, UK) which was fitted with a preparative scale column with a volume of 912.5 mL, an I.D. of 4 mm, a β-value range of 0.58–0.873, and a revolution radius of 110 mm.

Pilot scale experiments were performed on a Dynamic Extractions Maxi™ instrument (Slough, UK) which was fitted with a column with a volume of 18,000 mL, an I.D. of 10 mm, a β-value range of 0.54–0.924, and a revolution radius of 300 mm.

2.4. RP-HPLC purification

2.4.1. Sample preparation

The sample solution was prepared by dissolving the crude spinetoram (0.25 g/mL) in acetonitrile and filtering the solution through a 0.45 μm membrane syringe filter (Whatman, 25 mm, GD/X, PTFE).

2.4.2. Analytical RP-HPLC

Isocratic elution was performed using a mobile phase consisting of 10 mM ammonium hydroxide/acetonitrile (5:95, v/v) at a flow rate of 1.0 mL/min. The injection sample size was 20 μL and detection was by UV absorbance at 254 nm. This method was used for the analysis of both the feedstock material and the fractions generated during preparative chromatography.

2.4.3. Preparative RP-HPLC

The isocratic mobile phase comprised, 40:60 (v/v), 10 mM ammonium hydroxide/acetonitrile pumped at a flow rate of 800 mL/min. The sample injection volume for each run was 10 mL which contained 2.5 g spinetoram. Fractions were auto-collected by the Empower software according to absorbance at 260 nm. Stacked injections were performed at 8-min intervals. After every 36th run, the column was washed at 800 mL/min with 100% acetonitrile for 30 min and then re-equilibrated with the mobile phase.

The collected preparative scale HPLC fractions were analysed by RP-HPLC. Fractions that were >98% pure at 254 nm were pooled. Fractions of <98% purity were pooled and recycled once by preparative RP-HPLC. The pooled fractions were concentrated under reduced pressure by rotary evaporation to remove most of the acetonitrile to produce an opaque aqueous suspension which was extracted with dichloromethane. The dichloromethane extract was evaporated under reduced pressure by rotary evaporation to yield the purified components as white solids.

2.5. HPCCC purification

2.5.1. RP-HPLC analysis method

The following RP-HPLC method was used for the initial analysis of crude spinetoram and for the analysis of preparative HPCCC derived fractions. Gemini NX, 4.6 mm × 150 mm, 5 μm, C18 column, solvent A: 0.1% (v/v) TFA in water; solvent B: 0.1% (v/v) TFA in MeCN, flow rate 1 mL/min; gradient 75–100% B/4 min; hold at 100% B for 2 min then 4 min at 75% B.

2.5.2. General conditions for all analytical, semi-preparative and preparative HPCCC separations

Solvent systems were prepared according to the empirical ratio desired and the phases were equilibrated and separated prior to use. The HPCCC column was filled with the upper, stationary phase. A 240 × *g* centrifugal field applied to the column and mobile phase was pumped into the centre of the column whilst the displaced stationary phase was collected from the peripheral column outlet. The volume of displaced stationary phase was used to calculate the stationary phase retention, S_f, for each separation. Spinetoram was dissolved in lower phase, typically 1 mL for analytical, 10 mL for semi-preparative, and 70 mL for preparative scale separations. Mobile phase flow rates were 1 mL/min, 6 mL/min, and 42 mL/min respectively. The separations were monitored by UV absorbance at 280 nm. S_f was 0.75 for the analytical scale, 0.82 for the semi-preparative scale (1 g loading), and 0.80 for preparative scale separations.

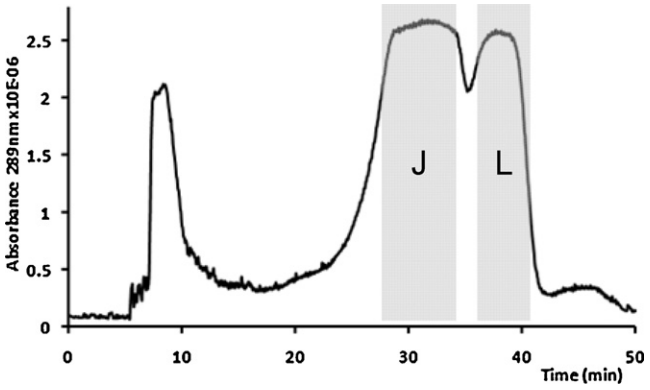

Fig. 2. Analytical scale HPCCC separation crude spinetoram using HEMW at (6:1:6:1). Conditions: sample loading 30 mg; upper phase stationary; column volume 22 mL; flow rate 1.0 mL/min; S_f 0.75; 1600 rpm; 30 °C; UV detection at 280 nm.

2.5.3. Conditions for pilot scale separations

The Dynamic Extractions Maxi™ CCC centrifuge (Dynamic Extractions, Slough, UK) is equipped with two bobbin-mounted coils which together form the 18,000 mL total column volume. The centrifuge can be rotated at up to 600 rpm (121 × *g*) and is connected to an Armen Industrial CCC Control Unit pumping system (Armen Instrument, Vannes, France) with in-built Knauer UV spectrophotometer and preparative flow cell (Knauer, Berlin, Germany).

The two separate coils which form the column were filled with stationary phase (upper phase) at 2000 mL/min in parallel. Instrument rotation was set at 600 rpm (121 × *g*) at a temperature of 30 °C. The coils were equilibrated in reversed phase mode (lower phase mobile) from head-centre to tail-periphery at 700 mL/min in parallel. Following equilibration, the flow was changed to serial through the two coils at 358 mL/min. S_f was 0.79 for the pilot scale separations. The sample was injected via a 920 mL manual sample loop, the UV signal was monitored at 289 nm and fractions were collected manually for analysis by HPLC.

3. Results and discussion

3.1. Initial solvent system selection and analytical scale HPCCC separation

For HPCCC, the use of the HEMWat solvent system was indicated when crude spinetoram exhibited good solubility in mixtures of hexane, ethyl acetate, methanol and water. Scouting of the lipophilic end of this series using simple test tube distribution studies of spinetoram indentified solvent system 25 in the HEMWat series (hexane: ethyl acetate: methanol: water, 6:1:6:1, v:v:v:v) which yielded distribution ratios for spinetoram-J and spinetoram-L of approximately 1. Analytical scale HPCCC separation of 30 mg of spinetoram using solvent system 25 in reversed phase mode, lower phase as the mobile phase, resulted in a separation of spinetoram-J (31 min) and spinetoram-L (38.5 min) roughly similar to that obtained using RP-HPLC (Fig. 2).

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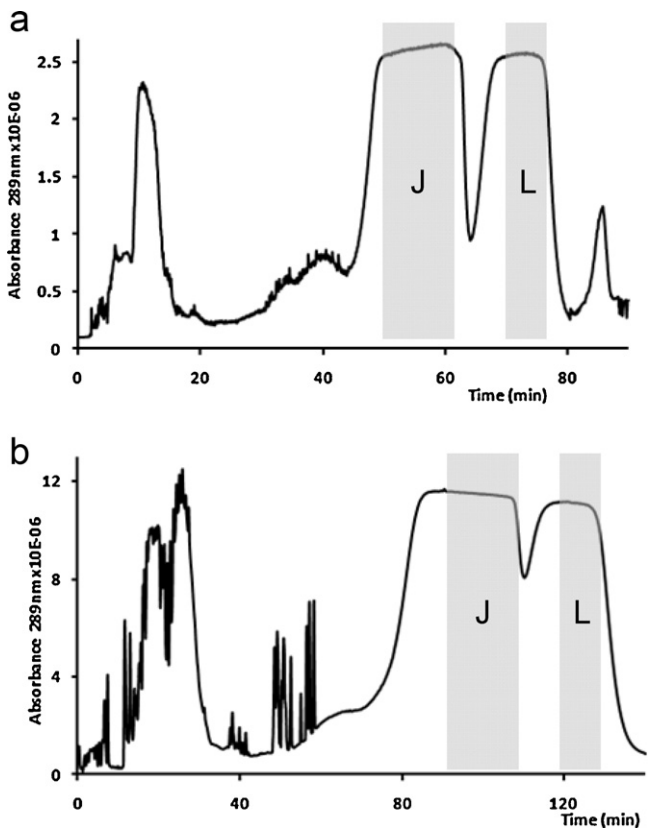

Fig. 3. Pilot scale HPCCC separation of crude spinetoram using HEMW at (5:1:5:1). (a) Semi-preparative scale front run. Conditions: sample loading 0.5 g; column volume 132 mL; upper phase stationary; flow rate 6.0 mL/min; S_f 0.82; 1600 rpm; 30 °C; UV detection at 280 nm. (b) Pilot scale. Conditions: sample loading 111 g; column volume 18,000 mL; upper phase stationary; flow rate 358 mL/min; S_f 0.79; 600 rpm; 30 °C; UV detection at 289 nm.

During this initial feasibility study, some investigation of the use of acidic and basic modifiers was undertaken: acidic modifiers were detrimental to the quality of the separation and when ammonia was used, it offered no significant advantage. All further development and scale up work was performed without the use of pH modifiers and by the use of reversed phase (RP) elution.

3.2. Optimisation of large scale separation conditions and loading

The goal of the separation optimisation studies was to maximize spinetoram loading, therefore, throughput and yield, without losing resolution of components spinetoram-J and spinetoram-L for the 18 L pilot scale HPCCC chromatography. Scale up of HPCCC separations has been demonstrated to be linear on identically designed columns [8]. Further development of this separation was performed on a semi-preparative scale column with a volume of 132 mL which is representative of a 136-fold scale-up to the 18,000 mL column.

In order to improve the resolution of components spinetoram-J and spinetoram-L, and therefore, increase the loading and throughput obtained at the analytical scale, the more polar solvent system, HEMWat 23 (hexane/ethyl acetate/methanol/water, 5:1:5:1, v:v:v:v), was employed. This led to increase of separation time but still provided higher throughput due to better resolution between the target compounds. Separation of 500 mg of spinetoram using HEMWat 23 is shown in Fig. 3a. Further increase in loading up to 1 g without compromising purity and yield was possible.

The semi-preparative separation was scaled up to a preparative column with a volume of 912.5 mL, giving a scaling factor of ×7. Using the same solvent system, both the sample loading and mobile phase flow rate were increased 7-fold, to 7 g and 42 mL/min respectively. Based on HPLC analysis, HPCCC fractions meeting the purity specification of >98% were combined and concentrated to dryness. The resulting mass balance data from this separation are shown in Table 1.

3.3. Pilot scale column HPCCC separation

Currently, the largest available HPCCC column has a volume of 18,000 mL representing a scale factor of ×19 from the preparative column above [12]. Theoretically and practically, the scaled loading on the pilot column would have been 136 g per injection. However, in light of the experiments on the preparative column which produced 15 wt.% of mixed fractions to be re-purified, a conservative approach was adopted and the material was purified in batches of 111 g/injection. In order to further increase resolution, the mobile phase flow rate was lowered to 360 mL/min. Typically, spinetoram-J (>98% purity as specified) was collected between 90 and 110 min and spinetoram-L (>98% purity as specified) between 120 and 130 min. A representative chromatogram is shown in Fig. 3b.

Eluate collected between 111 and 119 min, representing mixed fractions, was pooled with the equivalent fractions from eight more runs. Solutes from this pooled material were re-purified in a total of another 4 runs which allowed isolation of more pure components. Impure fractions from these four runs were combined and purified in a fourteenth and final run.

The final recovery of spinetoram-J and spinetoram-L, expressed as weight of target compound recovered from the initial 1 kg of spinetoram, was 63% (93% of theoretical) and 15% (63% of theoretical) respectively. The purities of the final products spinetoram-J and spinetoram-L were 97% and 98% respectively.

3.4. Preparative RP-HPLC separation of spinetoram

The isocratic analytical and preparative RP-HPLC chromatograms of spinetoram are shown in Fig. 4. The major component is spinetoram-J followed by spinetoram-L. The preparative RP-HPLC chromatogram resulted from a 2.5 g injection of spinetoram and a total of 400 runs were required to process 1 kg of spinetoram. The spinetoram-J fractions were obtained in >98% purity in a single pass. The spinetoram-L fractions were obtained in 92% purity from single pass preparative HPLC and were recycled once to obtain >98% purity. This required an additional 60 HPLC runs. The HPLC fractions contained too much water (40% aqueous) to evaporate directly to isolate purified components so the acetonitrile was removed by evaporation and the organic solutes extracted from the remaining aqueous layer with dichloromethane. The dried, dichloromethane solution was evaporated to render the purified components as white solids.

3.5. Comparison of RP-HPLC and HPCCC separation methods for 1 kg spinetoram processing

The purity and yield of spinetoram-J and spinetoram-L from 1 kg of crude spinetoram using each method was virtually identical although the methods required different numbers of re-processing runs of mixed fractions to achieve this. Thus, direct comparison of the performance parameters for 1 kg spinetoram RP-HPLC and HPCCC campaigns are particularly relevant and are summarised in Table 2.

As can be seen in Table 2, there is an inverse relationship between the total runs required for each campaign and the sample loading per run. RP-HPLC required approximately 33 times the number of runs whilst the loading per run was 44-fold less than

Table 1
Mass balance and fractions' purity of 7 g spinetoram HPCCC preparative separation.

Fraction	Start (min)	End (min)	Total time (min)	Fraction volume (mL)	% J purity by HPLC	% L purity by HPLC	Mass (g)	% Yield
4	40	48	8	320	12			
5	48	62	14	560	99		3.39	48.4
6	62	66	4	160	93	6	1.05	15.0
7	66	69	3	120	13	86		
8	69	81	12	480		99	1.12	16.0

Table 2
Comparison of performance parameters of HPCCC and RP-HPLC chromatography for 1 kg spinetoram separation campaigns.

Parameter	HPCCC	RP HPLC	Ratio HPCCC/RP-HPLC
Run time/injection (min)	140	8	17.5
Mobile phase flow rate (mL/min)	360	800	0.45
Operating pressure (psi)	220	500	0.44
Sample loading/run (g)	111	2.5	44.4
Dynamic loading (g/injection/L column volume)	6.17	1.05	5.88
Injection volume (mL)	920	10	92
Injection volume (% of column volume)	5.5	0.4	13.75
Total runs/kg	14	460	0.03
Time/kg (h)	22.0	53.3	0.4
Sample throughput (kg/h)	0.045	0.019	2.4
Total mobile phase used (L)	490	2560	0.191
Total stationary phase used (L)	243	N/A	
Total solvent used (L)	733	2560	0.29
Total fraction volume containing pure spinosyns (L)	86	1196	0.07
Recovery J (g/%)	627/63	630/63	1
Purity J (%)	97	97	1
Recovery L (g/%)	146/15	150/15	1
Purity L (%)	98	98	1

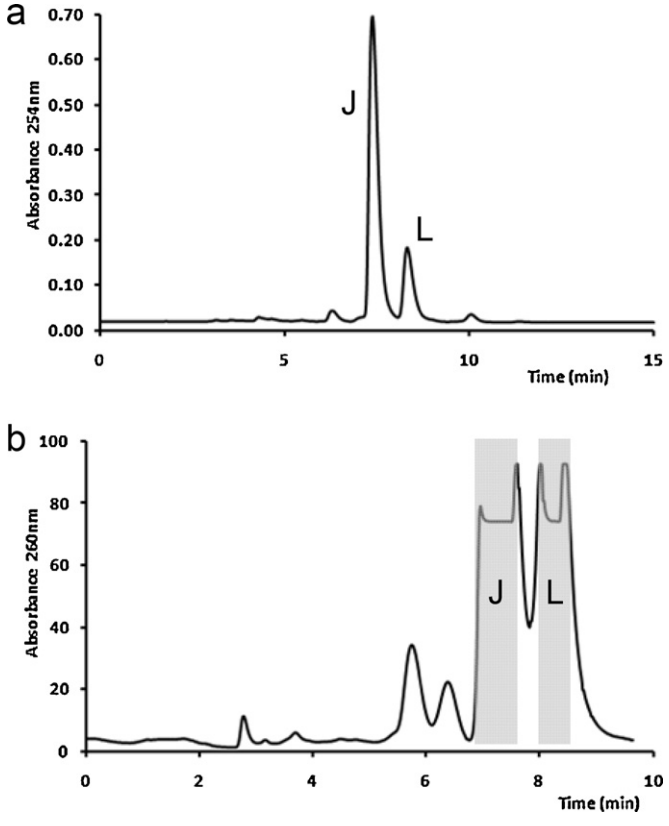

Fig. 4. Typical isocratic RP-HPLC separation of spinetoram. (a) Analytical scale. Conditions: Phenomenex Gemini C18, 4.6 mm × 250 mm, 5 μm; 1.0 mL/min; mobile phase 10 mM ammonium hydroxide/acetonitrile (5/95); UV detection at 254 nm. (b) Preparative scale. Fractions combined for spinatoram J and L are highlighted: Conditions: Waters Xterra C8, 11 cm × 25 cm, 10 μm; 800 mL/min; mobile phase 10 mM ammonium hydroxide/acetonitrile (40/60); sample loading 2.5 g; UV detection at 260 nm.

for HPCCC. This can be directly correlated to the different volumes of active stationary phase available in each separation method. The stationary phase in HPCCC is a liquid and typically comprises 75% or more of the column volume. The lack of a solid support means that this entire volume is accessible to solute. This contrasts with the relatively low active stationary phase volume of RP-HPLC supports and allows the injection of up to 10% or more of the column volume without chromatographic disturbance.

Product throughput involves both the processing time for the crude spinetoram, any re-processing of mixed fractions, and post-run product recovery. The initial crude sample throughput was approximately 2.4-fold higher for the HPCCC separation despite the longer individual run time. Whilst not directly factored into this analysis, the re-processing of the mixed HPCCC fractions involved 5 additional runs. For RP-HPLC, the later-eluting component, spinetoram-L, required 60 re-processing runs.

Following chromatography, product recovery effort is directly related to the volume and the water content of the target fractions. The volume of the target containing fractions was approximately 14-fold higher for RP-HPLC. HPCCC fractions, 86 litres, contained approximately 17% (14.6 L) of water, with the other 83% comprising volatile organic solvents whilst the 1196 L of HPLC fractions contained 40% or almost 480 L of water. The outcome was a relatively simple evaporative workup for the HPCCC purified material but for RP-HPLC, direct evaporation was prohibitive because of this large volume of water. Instead, the HPLC workup required evaporative removal of the acetonitrile followed by extraction of product into dichloromethane, drying and evaporation. In short, the HPCCC fraction work-up was faster, far less labour intensive and far less energy consuming than that required for the HPLC process.

The HPCCC process consumed less than 30% of the total solvent used for RP-HPLC, 733 L versus 2560 L. Of these volumes, organic solvents comprised 88.5% (648 L) and 60% (1536 L) for HPCCC and RP-HPLC respectively. For HPCCC this can be further broken down into 235 L hexanes, 75 L ethyl acetate, and 337 L

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methanol. Even assuming equal cost per litre of solvent the HPCCC cost for solvent is half that of RP-HPLC. Furthermore, this reduced solvent usage translates directly into lower waste-solvent disposal costs.

4. Conclusions

This study represents the first reported head-to-head comparison of pilot scale high performance countercurrent chromatography with an alternative separation method. Several pilot scale countercurrent separations have been described in the literature but the purpose of these studies was to demonstrate the scalability of the technique. It is clear from this work that HPCCC can offer significant solvent cost savings. Higher sample throughput using HPCCC was also obtained with respect to both crude sample processing and post-run processing.

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