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Use of ultrasound to monitor the packing of large-scale columns, the monitoring of media compression and the passage of molecules, such as monoclonal antibodies, through the column bed during chromatography

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Abstract

The novel use of ultrasound as a detector in pilot- and production-scale chromatography is described. The difficulties in packing production scale chromatography columns using an integral packing valve are reviewed. Results are presented from the packing of 400- and 600-mm diameter columns with various medias. From these results it is proposed that when packing large columns using a packing valve, for a given medium and column size, there is an ideal rate and pattern (or control ''corridor'') by which the bed builds in order to give optimum performance. Ultrasound was shown to be able to monitor the building of such a column bed as the medium was pumped into the column. It was found that the ultrasound detector was sensitive to bed compression, mobile phase composition and components such as acetone, albumin, casein and monoclonal antibodies while on the chromatography bed. This enabled the visualisation, by ultrasound, of these components as they were chromatographed through the column.

Keywords: Preparative chromatography; Ultrasound techniques; Monoclonal antibodies; Protein A

1. Introduction

The packing of pilot- and production-scale chromatography columns has been primarily carried out as described by Janson [1]; by pouring a 10–50% homogeneous slurry into the column—open at one end—with an extension tube to accommodate the extra volume. Mobile phase is then passed through the column at gradually increasing flow-rates in order to obtain a homogeneous bed. The piston is then replaced and made to compress the bed by about 5 mm.

Ladisch [2] described a method widely used for production-scale silica columns whereby the slurry is pumped into the column with a fixed frit at the outlet end. A hydraulically powered piston is pushed into the open end and then hydraulically moved along the column axially compressing the slurry forcing out the excess slurrying liquid. In such a way the bed is packed. Such packing is called ''axial compression'' and if the piston is left in place under constant force it is called ''dynamic axial compression''.

A third method, described in 1998 [3] and patented in 1996 [4–6], uses a packing valve to

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access the inside of the column instead of removing the piston or any axial plate. In this case valves located in the axial piston and fixed end plate allow slurry to be pumped into the column void. Media builds up against the exit mesh (frit) while the slurrying liquid leaves through the mesh (or sinter, also called ''frit'')

This paper deals with columns that are packed using such a packing valve. These columns have the advantages of convenience and safety in that only valves are used to pack and unpack, no lifting gear is required to move the piston or end plate, a reduction in the level of expertise seems possible and the labour time is greatly reduced.

Production-scale columns with packing valves were introduced in 1995 with sizes from 100 mm to 2 m diameter. At present over 1000 such columns are being commonly used worldwide in the biopharmaceutical industry [7].

Packing large diameter columns (above 400 mm, above 20 l bed volume) by removing the piston and pouring the slurry in, is compared to using a valve to access the interior of the column in Table 1. Although the introduction of a packing valve has made packing production scale columns significantly easier it has also introduced its own unique problems and made some previously robust parameters more sensitive. These are mainly on controlling a new set of variables that come with the use of the packing valve. Table 2 itemises these new parameters along with those ones that have become more sensitive to the final efficiency of the packing valved columns.

The packing of large-scale columns is a skilled job requiring training and experience. A packing valve helps to achieve consistent results, but only experience and time-consuming testing of trial packs can indicate the optimal slurry concentrations and pump pressure versus flow profiles for a given media in a given column. Also, many columns are steel and the operator can have little idea of what is happening to the medium inside during packing.

This article proposes that the rate and pattern of bed height versus time during packing packing-valved columns can be used as the controlling criterion thereby obviating the pressure, flow and concentration of the slurry parameters itemised in Table 2.

The investigation of the use of ultrasound to determine packed bed position and compression during and after packing is described. Periodic ultrasound vibrations were axially pulsed through the column and the reflected returning signal was analysed. The information obtained from the ultrasound

Table 1
Differences between packing by opening the column and pouring slurry in versus pumping slurry in using a valve

Point	Opening the column by removing the top plate or piston	Accessing the interior of the column using a packing valve
1	Total amount of media is put into the column at the beginning of packing	Media is pumped in as part of the packing process and thus is continually increasing in amount and concentration during the pack
2	Ratio of slurry to packing liquid is constant within the column during the pack	Ratio of slurry to packing liquid is continually changing within the column during the pack
3	Packing pressure changes due to the bed being hydrodynamically or mechanically compressed.	Packing pressure changes due to continually increasing the bed height and amount of media in the column
4	Bed height only varies between the settled and compressed value	Bed height is continually increasing throughout the pack
5	Column can be packed using a constant flow or constant pressure method	Since the bed height and thus the column is continually changing the pressure versus flow graph is also changing —this makes packing at a constant pressure or constant flow difficult

Table 2
Important parameters to consider when packing columns with packing valves

Point	Parameter
1	Slurry concentration in slurry tank
2	Slurrying liquid–buffer concentration, viscosity
3	Temperature of slurry and priming liquid in column
4	System characteristics-back pressure from system
5	Volumetric flow-rate of motive air to packing pump-motive air pipework
6	Bed support (frit) material and pore size e.g. sintered polyethylene, sintered polypropylene, and woven steel mesh

signal was also interrogated to see if it showed the position of chromatographing species. A production purification of a monoclonal antibody on a protein-A column was monitored in such a way.

The use of ultrasound in chromatography appears to be sparse. It has been specifically used in ion-exchange columns that are used with a supernatant free of media; in other words, the packed bed fills the column partially with a liquid layer lying on top of it. In such cases an ultrasound emitter/receiver is used as a level sensor on an ion-exchange column [8] The advantage here being that the ultrasound is not scattered by any supernatant fines, as would be the case if using an optical device. The ultrasound transducer would be placed above the top of the bed looking down onto the bed–supernatant interface. A similar patent [9] whereby the top ultrasound sensor is supplemented by sensors on the side (lasers and photoelectric options are noted) has been described. This patent describes a ''top-spaced'' ion-exchange column where the media-free top space must be kept between 10 and 20 cm. The media described shrinks in high salt. When this bed shrinkage is detected more liquid is pumped in to retain the 10–20 cm media-free top space.

In 1980 Rosencwaig [10] filed a patent describing the use of ultrasound to detect components in the eluate of a chromatography column. The apparatus described is similar to a double beam spectrophotometer whereby there is two flow cells. Through one cell flows the pure mobile phase that has seen neither sample nor media. Through the other cell flows the eluate from the column. A piezoelectric device fires 30 MHz through both the cells; a piezoelectric detector at the opposite side of the flow cells detects any differences in amplitude received from the two cells. The flow cell pathlength is between 0.1 and 1 cm, similar to spectrophotometer cell sizes.

Finally an international patent was filed in July 2000 by the author describing the basis of these experiments, apparatus and ideas presented in this article [11].

2. Experimental

2.1. Apparatus and materials

The chromatography columns used were 400 and 600 mm diameter, 100–300 mm bed height, acrylic tubed 3 or 5 bar, Euroflow (Resolute) columns, manufactured by Euroflow, Brimscombe (Stroud, UK). An Amicon 180 mm diameter glass tubed Moduline II column was provided by a Euroflow customer. Almatec (UK) or Graco Husky (Belgium), double diaphragm air driven pumps were used to pump the slurry and cleaning solutions. All reagents were of analytical or spectroscopic grade from Fisher (Loughborough, UK) or Sigma. (Poole, UK). Water was treated by a 10-μm filter, followed by a 5-μm carbon filter, reverse osmosis, then deionised to 18.2 MΩ and 0.2-μm filtered and chilled to 15–17 °C. Batches of 500 l of 0.025 *M* Tris–HCl buffer were prepared as follows: 2 kg of tris(hydroxmethyl)aminomethane hydrochloride (Fluka No. 93347) were dissolved in 500 l water (18.2 MΩ 0.2-μm filtered); 3 l of 1 *M* NaOH was added giving a pH of ~7.4.

Tosoh BioSep kindly provided ToyoPearl 650 M; Merck (USA) kindly provided Fractogel EMD COO^-; BioSepra (France) kindly provided Ceramic

Hyper D; rmp Protein A Sepharose Fast Flow, Sepharose 4 Fast Flow and Q Sepharose 6 Fast Flow were kindly provided by Euroflow customers. All media were defined in packing buffer between 1 and 3 times by stirring in a 250-l polyethylene tank then left for between 1 and 18 h (depending of visual evidence of fines) and the supernatant vacuumed off.

Hammersten casein solution was prepared by dispersing 10 g of Hammersten casein in 500 ml water. A white suspension was obtained with a pH of 4.9. On adding 20 ml of 1 *M* NaOH and making up the volume to 1 l with water the pH went to 10.3. On standing overnight the solution remained translucent without a precipitate. This method was obtained from Ref. [12].

The pharmaceutical production facilities for the production and purification of monoclonal antibodies were kindly shared with us for these experiment by a Euroflow customer. An Amersham BioProcess System was used to dilute and supply buffers and samples to the column and to collect the fractions.

Agfa NDT (Lewistown, PA, USA and the University of Warwick Science Park, Coventry, UK) kindly provided the ultrasound equipment.

2.2. Methods for packing large columns

Packing columns with packing valves is described in Ref. [3]. In brief, the column and all lines are firstly fully primed with water or buffer, particular care is needed to ensure no air remains under the bed supports (meshes or sinters) or on the top mesh inside the column. Slurry is recycled through the valve, with the valve in the clean in place-running-retracted position whereby media is isolated from the column interior. Inserting the tube carrying the slurry into the column and then opening the opposite end mobile phase starts the pack. At this point the slurry then is pumped into the liquid filled column and media builds up on the opposite bed support to the inserted nozzle. The slurrying liquid leaves through the mesh or sinter while the media is held back since it is too large to leave via the mesh or sinter holes. The packing flow-rate is generally 300 to 500 cm/h. Once the column is deemed packed the valve is then changed to the clean in place (running) position; in this position the slurry lines are completely isolated from the column bed and mobile phase. Cleaning solution is pumped through the valve clearing the lines of slurry. The column is now ready to use.

This laboratory uses three different methods to pack large columns depending on the media as follows:

2.2.1. Packing method 1—pressure control

Fixing a Bourdon gauge on the mobile phase port at the end where the media is being pumped in monitors the pressure inside the column. The use of pulse dampeners on the slurry pump greatly enhances this technique. By observing this gauge one can watch the pressure rise as the bed height increases. The pressure then plateaus, as the column appears full. Then the Bourdon gauge shows a clear drop in pressure, ‘‘a dip’’, this is the indication that the column is sufficiently packed. At this point the nozzle is retracted and the column is deemed packed.

2.2.2. Packing method 2—packed bed-front control

The second method used continues as the first described above except the packed bed front is carefully watched. When this ‘‘front’’ reaches the adaptor (piston) ring or the top of the column internal bed height (on a fixed bed height column)—this marks the end of the pack, at this point the nozzle is retracted. A derivative of this, for certain medias, the end-pack criterion is the point when a clear band of liquid, containing very little media forms between the packed bed front and the adaptor (piston) ring.

2.2.3. Packing method 3—measured media control

The third method, often used in development, a measured amount of settled bed volume is transferred to a slurry vessel. This amount is the volume of the column bed required multiplied by the compression factor, defined as in row 1 in Table 3. This measured volume is all pumped into the column. The leaving slurrying liquid is recycled back to the slurry tank for the remaining 10% of the slurry. This is to ensure that all the media measured goes into the column. The bed is then flowed up and down to condition.

Table 3
Equations used

Eq. no.	Parameter (units)	Calculation
1	Compression factor, Cf (unitless) (note: this is not the SI unit of ''compression'')	(Settled media volume)/ (packed bed volume)
2	*N*, Number of theoretical plates in the column itself (unitless) (V_e = Peak elution volume or mm of chart $W_{1/2}$ = Peak width at 1/2 height in volume or mm of chart)	$(V_e/W_{1/2})^2 \times 5.54$
3	HETP, height equivalent to a theoretical plate (in units used for *L*) [*L* = bed height (cm or mm or μm)]	L/N
4	Plates/m, plates per metre (unitless) (if plates in μm)	1 000 000/HETP
5	Plates/m, plates per metre (unitless) (if plates in cm)	100/HETP
6	RPH or *h*, reduced plate height (unitless) (HETP and particle diameter are in the same units of length, e.g. cm)	HETP/particle diameter
7	Af, asymmetry factor (unitless) (From apex to baseline drop a perpendicular line, draw a horizontal line 10% the above baseline *a* = width of 1st half of horizontal of peak at 10% above baseline; *b* = width of 2nd half of horizontal of peak at 10% above baseline)	b/a
8	Velocity (cm/h)	[(ml/min) × 60]/ (cross-sectional area, cm^2)
9	Flow (ml/min)	(cm/h) × [(cross-sectional area, cm^2)/60]
10	Flow (l/min)	l/h × 16.7
11	Compression (%)	[(Settled bed volume/ packed volume) × 100)] − 100

2.3. Calculations

The test was performed using the parameters as laid out in Table 4 and the height equivalent to a theoretical plate (HETP) and asymmetries were calculated as described in Ref. [13] and are summarised in Table 3.

Table 4
Parameters used during testing packed columns

Parameter	Value
Linear flow-rate	20 to 30 cm/h
Volume of test injection	1–2.5% of column bed volume
Sample (probe solution)	1% acetone in the mobile phase (water)
Detection wavelength	280 nm UV
Detector sensitivity	At pathlength of 5 mm, 0.2 AU, FSD (absorbance units, full scale deflection)
Peak height	25–75% full scale deflection obtained

2.4. Packing Sepharose 4 and 6 Fast Flow

Sepharose 4 Fast Flow was packed into a 600-mm diameter using packing method 3 to give packed columns of volumes of 42 and 45 l—150 and 160 mm bed heights. The final packing pressure was varied between 0.4 and 1.8 bar. The priming and slurrying liquid was water or 0.1 *M* NaCl. Multiple packs were carried out.

Sepharose 6 Fast Flow (Q) was packed at a constant 3 bar pressure using packing method 2 into a 400-mm diameter column to 150 mm bed height.

The priming and slurrying liquid was water or 0.1 *M* NaCl. Multiple packs were carried out.

2.5. Packing ceramic Hyper D

Q Ceramic Hyper D F was packed into a 400-mm diameter, 200-mm bed height column. The priming and slurrying liquid was 0.025 *M* Tris–HCl buffer, pH 7.4. Multiple packs were carried out, slightly differently as to packing methods 1–3, as follows:

The medium was defined three times before each packing at ca. 30% slurry concentration in 0.025 *M* Tris–HCl buffer. The slurry was adjusted to 50% settled media volume 50% slurrying buffer (0.025 *M* Tris–HCl, pH 7.4). The flow-rate used at the start was 1000 cm/h. Near the completion of the pack the flow pulsed between 300 and 900 cm/h. The pack was considered complete when the pump stalled out at 1.5 bar; this was after 3–4 min from appearing full.

2.6. Ultrasound experiments

A series of experiments were carried out to explore the use of ultrasound to detect the position of the packed bed front as it builds during packing. A 1-MHz piezoelectric transducer crystal was strapped to the exterior wall of the 400-mm diameter column at 100 mm up from the bottom level of the column. The piezoelectric transducer was fired at 1-s intervals across the column. The same transducer received the return signal. This signal was attenuated by its journey and the attenuation was plotted against time as the bed built below, past and above the transducer position or as components passed the transducer. In a similar experiment an array of 16 transducers was strapped to the column—see Fig. 1. The attenuation of each transducer as the bed or components rose past them was similarly plotted.

2.6.1. Sepharose 4 Fast Flow—location of bed by a single ultrasound transducer

A 400-mm diameter×250 mm Euroflow column with transducer was packed by pumping 50% Sepharose 4 Fast Flow slurry via the packing valve into the water-primed column. The bed built up on the bottom mesh as usual. The graph of ultrasound signal versus time was plotted

Fig. 1. 16 Transducer array attached to the 400 column.

2.6.2. Sepharose 6 Fast Flow—monitoring the complete bed by multiple ultrasound transducers

A 400-mm diameter×300 mm Euroflow column with transducer array (see Fig. 1) was packed by pumping 50% Sepharose 4 Fast Flow slurry via the packing valve into the water-primed column. The bed built up on the bottom mesh as usual. The graph of ultrasound signal versus time was plotted.

2.6.3. Fractogel by ultrasound

The 400-mm column with the single transducer at the 100 mm bed height position was emptied. The piston was taken out and a concentrated slurry of Fractogel was poured into the open column. The medium was allowed to settle overnight (20 h). The bed height of the settled bed was measured and the piston replaced avoiding introducing any air. Downward flow was increased in 1-l/min steps from zero to 6 l/min during which time the bed height was noted. At the same time the ultrasound signal was recorded. The flow caused the bed to compress

thereby changing the ultrasound signal the ultrasound signal.

2.7. Ultrasound and model analytes

Albumin, casein, and acetone were chromatographed at process scale and monitored by ultrasound as they passed through a column as ''models'' for real production target molecules such as biopharmaceuticals.

A 400-mm diameter Sepharose 4 Fast Flow column was run downflow with water at 1 l/min. A 1-l volume of 10 g/l egg albumin was injected onto the column. The ultrasound signal was recorded against time. The term ''sonochromatogram'' will be used from here on to denote a graph of ultrasound signal versus time. Similar to an UV chromatogram which plots absorbance versus time, the sonochromatogram is defined here as a plot of sound absorbance or more correctly sound attenuation versus time.

This injection was followed up with a further injection of 0.5 l of the 10 g/l egg albumin. The resultant sonochromatogram was recorded. An injection of 10 g/l casein (boiled Hammarsten) was also carried out.

The chromatography of acetone passing through the column was clearly observed by ultrasound using the multiple transducers array shown in Fig. 1. The acetone was run at HETP conditions as shown in Table 4.

2.8. Ultrasound and monoclonal antibodies

In this experiment the ultrasound detector was used in a true production situation during the purification of a monoclonal antibody.

2.8.1. Biosynthesis and clarification

A mammalian cell culture was fermented in a 500-l stirred tank fermentor. At the end of the fermentation the cells die and release their IgG (immunoglobulin G). The fermentation liquor was disc stack centrifuged giving the primary recovery liquid. This was then microfiltered through a ''Cuno'' depth filter then sterile filtered through a Millipore PVDF 0.2-μm filter to yield 200 l of yellow liquid for chromatography. This liquid contained 0.425 g IgG/l or in the total 200 l, 85 g of IgG.

2.8.2. Chromatography

A 180-mm diameter Moduline II glass column was packed to 150 mm bed-height with rmp protein A Sepharose 4 Fast Flow, this being 3.82 l. An Amersham BioProcess System was used to dilute and supply buffers and samples to the column and to collect the fractions. The chromatography mobile phases used to separate the IgG from clarified cell supernatant are detailed in Table 5.

2.8.3. Ultrasound

Euroflow laboratory-designed software was used to log the ultrasound data to a laptop computer. A single transducer was placed at the base of the column, see Fig. 2. In this position the ultrasound signal would ''sense'' changes at the elution portion of the column bed.

Table 5
Run 1-mAb and Run 2-mAb chromatography conditions used to separate IgG from clarified cell supernatant

Step	Mobile phase (150 cm/h; 640 ml/min)	Purpose	Column volumes (CVs)
1	6 *M* Guanidine–HCl (aq., pH≈4.6)	To clean the column prior to process	8
2	0.05 *M* Glycine–glycinate pH 8.4	Equilibrium buffer	10
3, Run 1	Clarified process sample	Load	62 l (16 CVs)
3, Run 2	Clarified process sample	Load	138 l (36 CVs)
4	0.05 *M* Glycine–glycinate pH 8.4	Wash	10
5	0.1 *M* Glycine (HCl) pH 3.5	Elute IgG	3
6	0.1 *M* Citric acid, pH 2.1	Regeneration (strip)	2

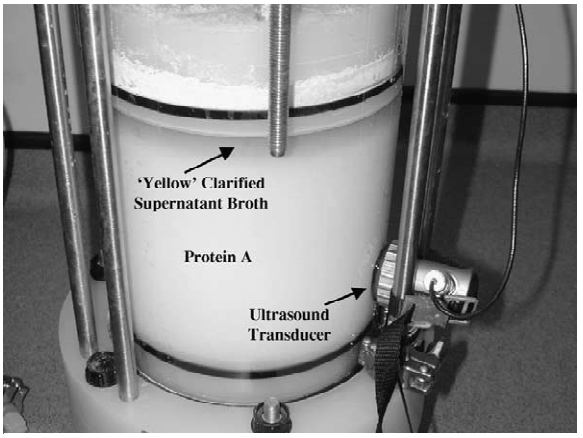

Fig. 2. Ultrasound transducer strapped to base of column—eluate side.

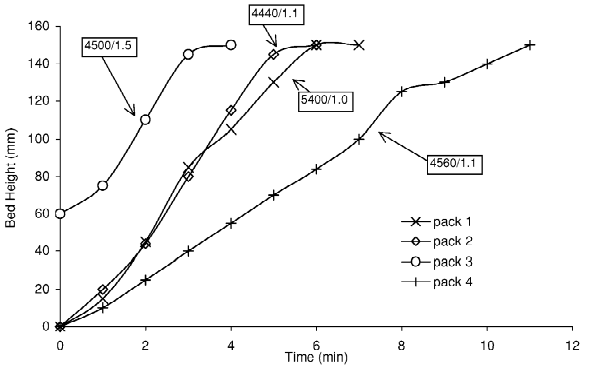

Fig. 3. Repeated bed building profiles of Sepharose 4 Fast Flow into a 600-mm diameter×150 mm bed height (42 l) Euroflow column. Results are shown as (plates/m)/(asymmetry).

3. Results and discussion

3.1. Important parameters in packing large scale valved chromatography

3.1.1. Columns

In 1994 the first columns large-scale columns were introduced which had valves with which to pack without removing the piston or lid [3]. Table 1 lists the main differences in the packing process between columns packed by removing the lid or piston versus packing via the valve. The gist of this table—and this work—is that when using a valve, the amount of medium in the column and the bed height is constantly changing during the packing. So, how can such columns be packed consistently and homogeneously, and what are the important control parameters.

Packing Sepharose 4 Fast Flow: The Sepharose 4 Fast Flow was packed at pressures between 0.4 and 1.8 bar. Fig. 3 shows the bed height versus time plots for four packs at various rates into the 600-mm diameter column. One of the packs—Pack 3—was actually started already half full with media which had been poured in and allowed to settle overnight. The efficiencies are displayed in Fig. 3. Note that all four results were good passes (rph less than 3 and Af 0.8–1.6). Table 6 reviews the results along with two others and shows that when the packing pressure is pushed too low as in the 0.4 bar pack, or too high as in the 1.8 bar pack, poor results with 4 Fast Flow were obtained. Not enough experiments have been carried out to state the exact pressure limits that would ensure a good pack with perfect confidence. However, from these results, a guide for packing 4 Fast Flow would be to stay in a ''pressure window'' of between 0.5 and 1.5 bar for column bed heights 100–300 mm.

Based on these and many similar results it is this author's view that the Sepharose 4 Fast Flow is a relatively deformable media and hence can bounce and morph to form well packed bed without ''remembering'' the trauma of the packing process. Thus in the case of Sepharose 4 Fast Flow the bed height build profile (e.g. those shown in Fig. 3) is less important than staying within the pressure ''window'' described above.

Packing Sepharose 6 Fast Flow: For the 6 Fast Flow medias, this laboratory has found that in order to force a linear bed height build, the bed must be packed at around 3 bar throughout the pack. Mean-

Table 6
Sepharose 4 Fast Flow packs

Pack no.	Final packing pressure (bar)	Plates/m	Reduced plate height	Asymmetry
1	1.0	5400	2.1	1.0
2	1.0	4440	2.5	1.1
3	1.0	4500	2.5	1.5
4	0.8	4560	2.4	1.1
5	1.8	2100	5.3	3.0
6	0.4	1140	9.7	1.6

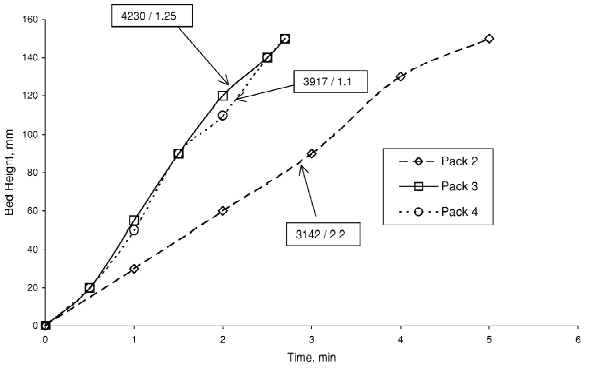

Fig. 4. Repeated bed building profiles of Q Sepharose 6 Fast Flow into a 400 mm diameter×150 mm bed height Euroflow column. Results are shown as (plates/m)/(asymmetry).

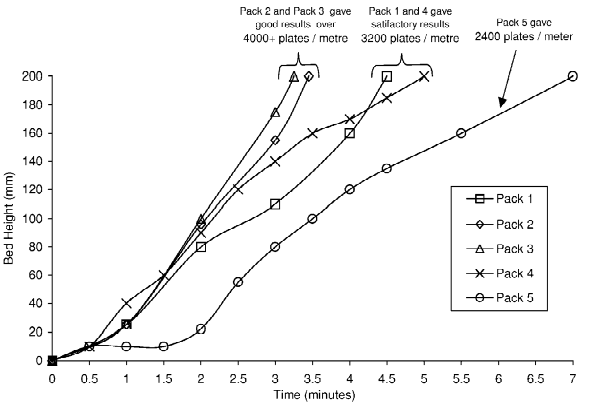

Fig. 5. Pattern of bed build for five ceramic Hyper D packs in the 400-mm diameter column.

while, as usual, the flow slowly reduces throughout the pack. When this is done the results are good, for example. reduced plate height of 2.4 (4230 plates/m) and an asymmetry of 1.25.

When the Sepharose 6 Fast Flow is packed at 3 bar but not throughout the pack, the resultant column takes longer to pack and appears to give poor asymmetries and plate counts. Fig. 4 shows the bed height versus time graphs for three packs. Two of these are in a tight corridor and gave good results shown on the graphs. A pack, with a bed building profile lasting 6 min, gave a tailing peak (asymmetry 2.2) with a second small broad tailing ''peak''. This effect was reproducible and is seen by many workers in this field. It is clear from Fig. 4 that one could draw control lines parallel to either side of the good packs and use this corridor to follow during packing the Sepharose 6 Fast Flow.

It is this worker's view that the Sepharose 6 Fast Flow being more rigid than the Sepharose 4 Fast Flow has to be built in a column homogeneously during the pack. Its rigidity leaves no margin for unsuitable bed building profiles.

Packing Ceramic Hyper D: Ceramic Hyper D is a very different media compared to the agarose Sepharose medias. In this particular experiment attention was applied to the stability and flow-rate optima. Hence for each of five packs carried out 12 HETP/Af tests were done along with an 18-h flow challenge to check the stability of the bed. Table 7 summarises the results.

Fig. 5 shows the bed building graphs of the five

Table 7
Test results from 5 BioSepra Ceramic Hyper D F packs; the flow-rate was 100 cm/h (2 l/min)

Pack no.	Results	Before stability downflow	Before stability upflow	After stability downflow	After stability upflow	Mean
1	Plates/m	3384	N/a	3100	N/a	3242
	Asymmetry	1.5	N/a	1.25	N/a	1.4
2	Plates/m	4881	3842	3857	3770	4088
	Asymmetry	1.4	1.4	1.4	1.3	1.4
3	Plates/m	4308	4328	4267	4147	4263
	Asymmetry	1.4	1.4	1.5	1.6	1.5
4	Plates/m	3485	3449	3142	3112	3297
	Asymmetry	1.4	1.4	1.6	1.8	1.6
5	Plates/m	2443	2161	2597	2398	2400
	Asymmetry	1.1	1.8	1.5	1.4	1.5

packs annotated with their results. It appears that there is a packing rate ''corridor'' of varying width that must be followed to achieve a ''good pack''. The actual unique packing rate versus time path taken could be viewed as a ''fingerprint'' of the packed bed. If this fingerprint path falls within the corridor then a ''good'' column should be formed. This was also the case suggested from reviewing the Sepharose 6 Fast Flow results (Fig. 4).

3.2. Use of ultrasound

These experiments were initiated simply by the need to ''see'' inside an 81-l steel column as it was packed with a potassium hexacyanocobalt(II)–ferrate(II) medium used for capturing radioactive caesium. Using a clinical ultrasound cardiac instrument the inside of the column could be viewed as the bed was built and consequently enabled success in packing such a difficult medium. Those packs are not described here but they were the genesis of the work described in this paper—that being the use of ultrasound to help pack large columns with packing valves.

3.3. Ultrasound and media

3.3.1. Single transducer ultrasound trace of the Sepharose 4 Fast Flow packed bed front

Fig. 6 shows the ultrasound trace as a column was packed with medium—initially the column only contained water and the signal is shown as 85%. The ultrasound emitter/receiver was strapped at the 100 mm level as usual. The insertion of the nozzle—at time 5 min—was detected using ultrasound despite that the nozzle is not directly in the ultrasound beam. The column then filled with medium and the ultrasound signal dropped to the 60% level at the 10-min point. The ultrasound emitter/receiver is a piezoelectric crystal of a finite size, about 20 mm in this case. At about 12 min, part of the crystal is covered by packed bed, part by slurry. This gradual change as the packed bed front climbs upwards across the crystal appeared to always cause this kind of inflection shown.

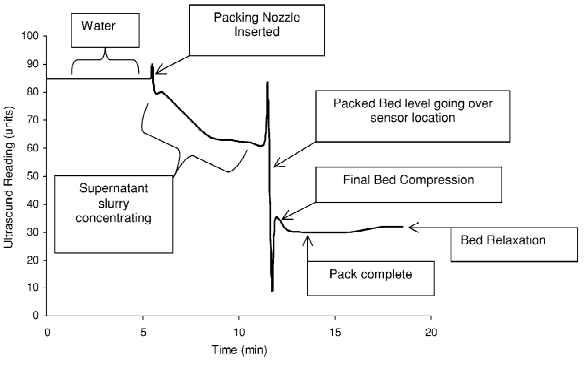

Fig. 6. Signal obtained from a single transducer located on the exterior wall of a 400-mm diameter column as it is packed with Sepharose 4 Fast Flow.

3.3.2. Multiple ultrasound transducer trace of bed building

Fig. 1 shows the 16 transducer array attached to a 400-mm acrylic column. This column was packed with Sepharose 6 Fast Flow to 300 mm bed height. Fig. 7 shows how the bed as it was built causes changes in the ultrasound signal as it passes each transducer. Thus we obtain multiple traces similar to the single trace obtained in Fig. 6. Transducer number 1 is at the bottom of the column, 16 at the top. Transducer number 2 was faulty and did not give a signal. Clearly with such an array the build pattern of these columns can be monitored by ultrasound. The signal also gives information about the compression of the media. Fig. 7 shows each vertical ''slice'' of the column to give a similar signal level (around 60). Thus the column appears by ultrasound to be homogeneously compressed. A HETP/Af test gave excellent results. Transducer number 16 is higher because the plastic packing tip—directly in its beam, still in the bed at this point—attenuates the signal. This shift in attenuation does not appear to affect the trace shape and could be subtracted to be directly comparable to the other transducers.

3.3.3. Use of ultrasound to directly measure fractogel bed compression

The relationship found between the ultrasound signal and the degree of compression is shown in Fig. 8. Clearly there is a direct linear relationship between compression of the Fractogel media and the ultrasound signal.

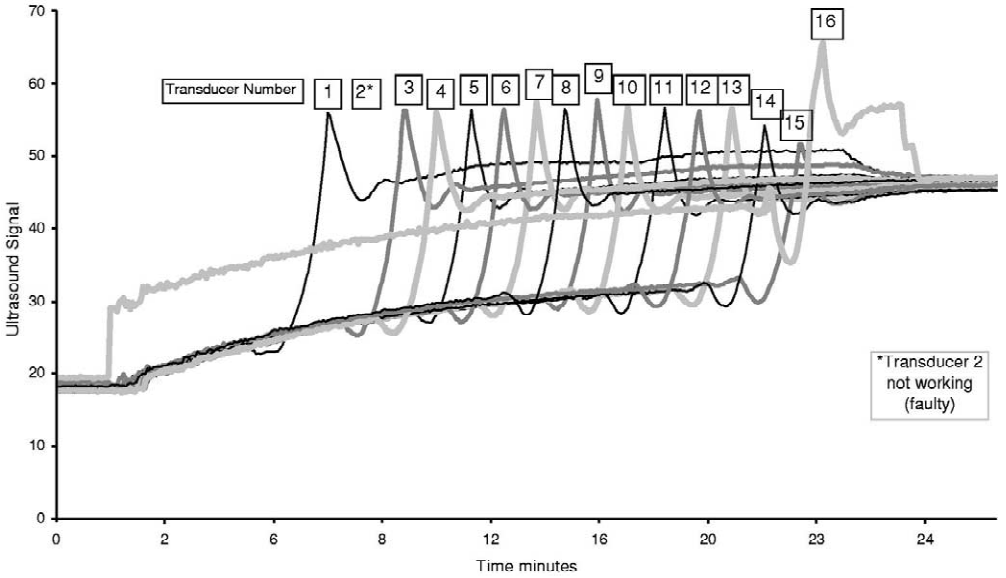

Fig. 7. Signals from 16 transducers monitoring the bed height build of a Sepharose 6 Fast Flow column, 400 mm diameter×300 mm bed height.

3.4. Ultrasound and analytes

3.4.1. Model proteins—albumin and casein

3.4.1.1. Ultrasound detection of albumin as it passes through a column

Fig. 9 shows two injections of egg albumin solution. The purity of the albumin used was a minimum 98% by agarose electrophoresis [14]. The first injection of the albumin was twice the load of the second. The sonochromatogram does appear to detect that the first load was significantly higher than the second, albeit not a direct linear relationship in this case.

Every time the egg albumin was injected this characteristic sonochromatogram seen in Fig. 9 was obtained. It was therefore thought that different proteins might have different sonochromatograms, due to their elution profiles as they pass through the medium or their own unique ultrasound transmission properties or a combination of both.

After each injection of the impure albumin was

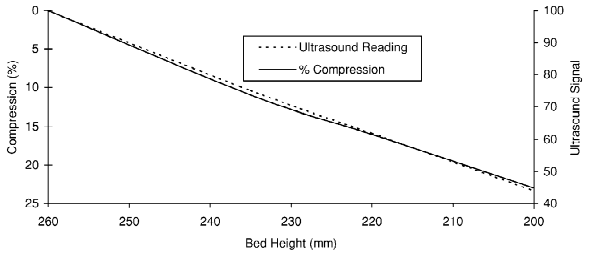

Fig. 8. Change in ultrasound signal in comparison to the actual compression value caused by the flow packing compression of Fractogel.

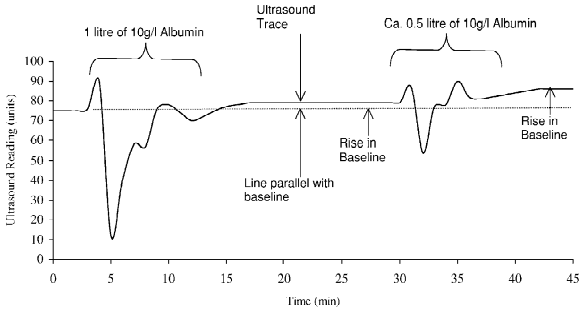

Fig. 9. Ultrasound trace of two injections of albumin at different volumes (loads) onto a 400-mm diameter×150 mm bed height Sepharose 4 Fast Flow column, the ultrasound transducer being at the 100 mm bed height position. The dotted line helps illuminate the rise in baseline upon subsequent injections of albumin.

carried out the baseline climbed to a higher constant level. It is suggested that this is due to fouling of the media due to bound species.

3.4.1.2. Ultrasound detection of two different proteins as they pass through a column

Fig. 10 shows the sonochromatogram of egg albumin and of casein passing through the Sepharose 4 Fast Flow column. It is noticeable that these sonochromatograms are very different. Chicken egg albumin (ovalbumin) is a phosphorylated glycoprotein. The peptide portion of the molecule has a molecular mass of 44 287 [15]. The carbohydrate and phosphate portions of egg albumin account for an additional 1428 and 160 g/mol, respectively [16]. Meanwhile, casein is a mixture of four proteins with molecular masses between 20 000 and 30 000. Two of these proteins (the α-caseins) are of a similar molecular mass—about 27 000 [17]. It is suggested that the sonochromatogram shows the expected elution profile of the caseins since it has three distinct ''peaks''; the first casein peak (the less well defined one) is the two α-caseins coeluting since they are of a similar higher molecular mass than the other two. These smaller two have molecular masses of 24 000 and 21 000 [17] and hence would elute as two separate peaks in order of decreasing size. It is thus suggested that the two later peaks are these two caseins. Also the casein elutions are later than the albumin, which would agree with the caseins being smaller molecules, and thus being retained longer on the gel-permeation column.

This suggests that the casein proteins are detected by the ultrasound as the pass by the transducer. The albumin trace may be more complex due to the albumin used being more than a single component species or even we are seeing temperature changes due to adsorption and desorption.

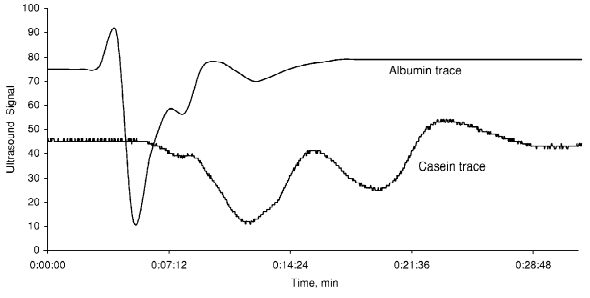

Fig. 10. Ultrasound trace of two injections of different proteins, one of albumin and one of casein onto a 400-mm diameter×150 mm bed height Sepharose 4 Fast Flow column, the ultrasound transducer being at the 100 mm bed height position.

3.4.1.3. Multiple ultrasound transducer trace of a chromatographing species

Fig. 11 shows a multiple array sonochromatogram of acetone passing through a 400 mm×300 mm bed height Sepharose 4 Fast Flow column. Transducer 16 at the column top shows the acetone band entering the column. Subsequent transducers map its travel. The traces get wider and shorter as the band goes through the column bed—as would be predicted until the final Transducer 1, shows a shorter wider band. The peak eluted was symmetrical and had good efficiency. It appears the live chromatographing of a species as it passes through the column can be visualised using such an ultrasound array.

3.4.1.4. Monitoring of monoclonal antibody (mAb) on a protein A column by ultrasound

Fig. 12 shows the sonochromatogram of Run 1-mAb as described in Table 5. At the start of the trace, before the 62-l loading begins to pass the ultrasound transducer, the 6 *M* guanidine–HCl is seen leaving the column, probably with other species. Then, as annotated on the graph the 62 l of process liquid starts to pass and the baseline stabilises. Then the ten column volumes of the pH 8.4 wash can be seen. Based on the previous compression experiments the rise seen in the sonochromatogram is perhaps due to the bed relaxing because the 0.05 *M* glycine–glycinate is likely to be much less viscous than the process liquid and thus would generate less back pressure. This was also evidenced by a gap appearing at the inlet end of the bed when the process liquid was loaded. This gap can be seen in Fig. 2, the yellow supernatant being the process liquid.

After the wash the elution of the IgG step occurs, it is not clear which peak is which at this point in the research. The citric acid regeneration step shows many small peaks and troughs that appeared to correspond to switching the column in and out of line thereby relaxing and compressing the bed.

Fig. 13 shows the concurrent UV and sonoch-

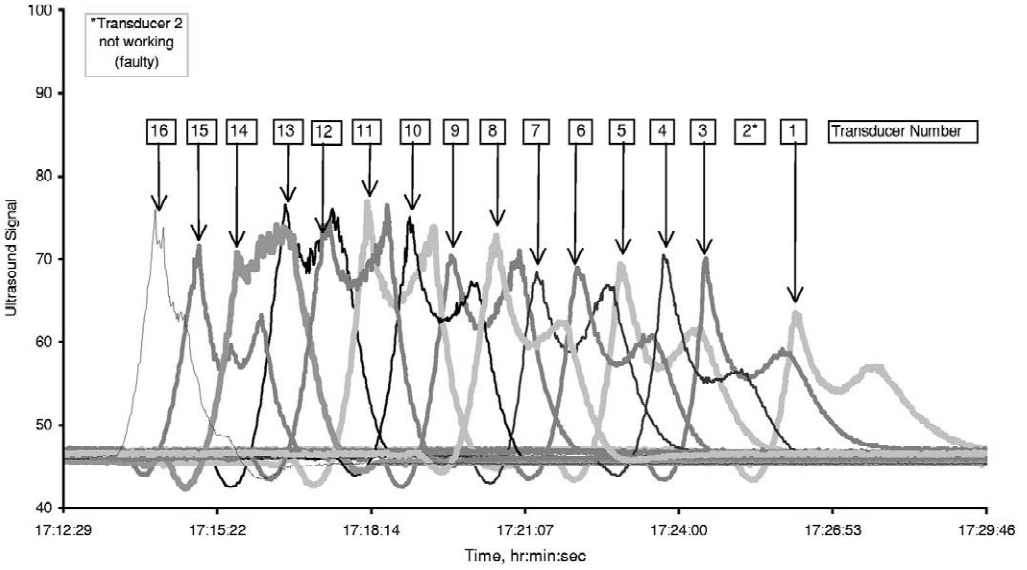

Fig. 11. Multiple ultrasound transducer array showing acetone as it passes through Sepharose 6 Fast Flow 400 mm diameter×300 mm bed height column; 1.5 l injection of 1% acetone; flow-rate 2 l/min.

romatograms for Run 1-mAb. The UV chromatogram shows a single peak, the IgG (labelled peak C). Meanwhile the sonochromatogram shows many peaks. Based on the research described in this paper it is proposed that the ultrasound peaks are a combination of bed compression and mobile phase constituent.

UV can only detect species with chromophores of high enough absorptivity to be detected at 280 nm. The buffers do not contain such chromophores and as such are invisible to the UV detector.

At point A in Fig. 13 the column was taken offline

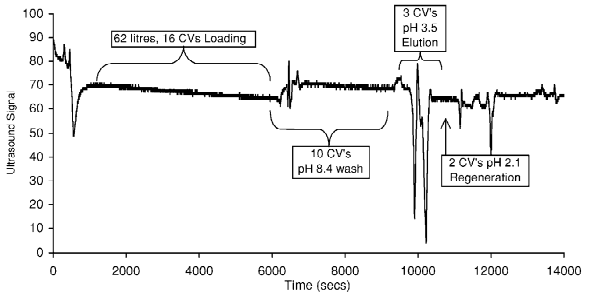

Fig. 12. Run 1-mAb, 62 l clarified process liquid on a Moduline II 180 mm column (CV=column volume).

in order to dilute buffer, the sonochromatogram suggests bed relaxation. At point B it goes back online and the sonochromatogram suggests recompression back to the previous level.

Peak A appears in both the blank and the process run. This peak appears rather large to be just due to bed compression. This suggests the peak is due to some material eluting at the interface between the pH 8.4 buffer and the pH 3.5 buffer.

Peak B does not appear in the blank, it is thus suggested that at point C the IgG is starting to be displaced by the lower pH buffer leading to its maximum concentration at peak B—the IgG itself.

It is important to note the time lag between—what is suggested—the sonochromatogram IgG peak (peak B) and the UV IgG peak (peak C). This would be because the ultrasound emitter/receiver is looking through the last 10 mm of the protein A bed while the UV flow cell is downstream—post-column.

The load in Run 1-mAb was 26.4 g IgG in 62 l. Run 2-mAb (Fig. 14) was about double this load, 58.7 g in 138 l and thus acted as a spike to confirm that the sonochromatogram was seeing the IgG. Fig. 14 shows both the UV (peak B) and the sonochromatogram peak (peak A) increasing in a similar way in size and splitting into a double peak. This is

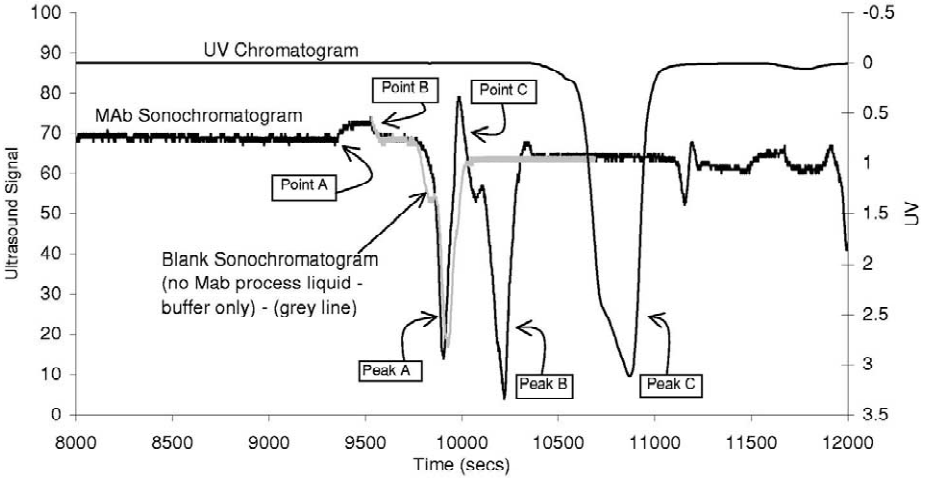

Fig. 13. Concurrent traces from the UV spectrometer with the ultrasound trace (sonochromatogram). Also shown is a separate run where only the buffer sequence was run, no process liquid was applied. This blank run is superimposed at the same process time point in the process run.

characteristic of this particular IgG process, thus confirming that the ultrasound is detecting the IgG.

4. Conclusion

The results from the packing experiments suggest that the unique build pattern of a chromatography bed directly relates to its resultant efficiency and asymmetry values. For softer gels like Sepharose 4 Fast Flow the control corridor that the bed-building pattern takes is a relatively wide corridor compared to Sepharose 6 Fast Flow where the bed build corridor is relatively tight or ''sensitive''.

For a ceramic media like Hyper D a more rigorous study was carried out which confirmed the idea of using the bed-building pattern as a criterion to control the packing process.

This series of studies revealed that using ultrasound, the presence of media in a column could be detected. The bed-building front could be clearly visualized by ultrasound and there was a direct relationship between ultrasound reading and bed compression.

Chromatographing components (acetone, egg albumin and casein) on the column were clearly visualized by ultrasound. Using a multiple array the actual chromatography of acetone as it passed through the bed was visualised and the band broadening could be seen. A process run of a monoclonal antibody was monitored by ultrasound giving a very detailed sonochromatogram. The sonochromatogram monitored the bed condition in terms of compression and presence of chromatography components as they passed through the column.

The author suggests that the high sensitivity

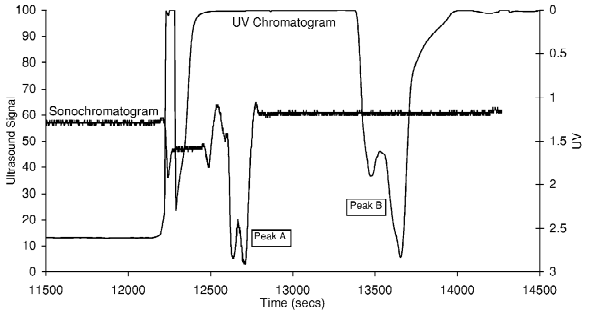

Fig. 14. Chromatogram of Run 2-mAb shows nearly double the load of that used in Run 1-mAb. This effectively acts as a ''spike'' to clearly identify which peak is due to the IgG. This peak splitting into a double peak is characteristic of this IgG process and is normally seen at such loadings.

shown by the sonochromatogram is due to the path length taken by the ultrasound pulse which is twice the column bed diameter—800 mm in the case of the 400-mm column—used in some of the experiments. This, in comparison to the 5- or 10-mm path length of an UV flow cell helps understand how such relatively small changes of solutes in the transmitting material can be detected by sound. The ultrasound does—based on the result of this study—have the advantage over UV absorbance in that it appears to behave as a universal detector. It can detect agarose concentration (Sepharose bed compression), polymethacrylate concentration (Fractogel), ceramics, simple salts, acetone and acid concentrations e.g. buffers and, of course proteins. It appears to be able to detect fouling on chromatography beds.

The sonochromatogram of the purification of a monoclonal antibody and the multiple array sonochromatogram of acetone showed what potential this technique can bring to production scale chromatography. It is thus suggested that ultrasound in production-scale chromatography has many additional functions compared to presently used detectors, these include:

(i) It is not in contact with the media or process liquids.
(ii) It can be used to monitor and thus aid control the building of a chromatography bed such that it stays in a proven control bed-build pattern.
(iii) It can detect when a column is fouled and perhaps not fit-for-use. In this case it could be used in production to determine whether to load a batch of process material onto the column or not, based on the preceding trend in the sonochromatogram; perhaps when the baseline reaches a predetermined level, this being learnt from previous experiences when columns failed during process when the sonochromatogram had hit a certain ''ceiling''.
(iv) Ultrasound can be used to measure the compression and expansion of the bed during chromatography.
(v) Using an array of ultrasound emitter/receivers the real-time chromatographic separation of species on the column could be monitored. This would give an insight into how species separate and also perhaps allow the monitoring of buffer exchange on the column.
(vi) It could also indicate that a clean-in- place (CIP) cycle is successful or whether it should continue on longer.

The value of ''seeing'' the chromatographic separation live in the bed is that it would locate the actually point in the bed where the target molecule was in relation to other species enabling better optimisation of bed height, buffer consumption, media usage and final target molecule concentration.

Further work being carried out on this subject includes the construction of a fully automated PLC/PC (Programmable Logic Controller/Personal Computer) control packing station that uses the 16 ultrasound emitter/receiver array to follow the build of the bed and allow the PLC to control this build within the control corridor. Also in development is the construction of a dedicated ultrasound detector that would be part of a chromatograph process system.

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