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A novel approach to improve operation and performance in flow field-flow fractionation

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ABSTRACT

A new system design and setup are proposed for the combined use of asymmetrical flow field-flow fractionation (AF4) and hollow-fiber flow field-flow fractionation (HF5) within the same instrumentation. To this purpose, three innovations are presented: (a) a new flow control scheme where focusing flow rates are measured in real time allowing to adjust the flow rate ratio as desired; (b) a new HF5 channel design consisting of two sets of ferrule, gasket and cap nut used to mount the fiber inside a tube. This design provides a mechanism for effective and straightforward sealing of the fiber; (c) a new AF4 channel design with only two fluid connections on the upper plate. Only one pump is needed to deliver the necessary flow rates. In the focusing/relaxation step the two parts of the focusing flow and a bypass flow flushing the detectors are created with two splits of the flow from the pump. In the elution mode the cross-flow is measured and controlled with a flow controller device. This leads to reduced pressure pulsations in the channel and improves signal to noise ratio in the detectors. Experimental results of the separation of bovine serum albumin (BSA) and of a mix of four proteins demonstrate a significant improvement in the HF5 separation performance, in terms of efficiency, resolution, and run-to-run reproducibility compared to what has been reported in the literature. Separation performance in HF5 mode is shown to be comparable to the performance in AF4 mode using a channel with two connections in the upper plate.

1. Introduction

Field-flow fractionation (FFF) is a well known family of separation methods that vary in the physical nature of the force field applied to generate separation [1]. Flow field-flow fractionation (F4) [2] is the most popular type of FFF; it employs a hydrodynamic cross-flow, and it exists in several variants: symmetrical F4 [3], asymmetrical F4 (AF4) [4], and hollow-fiber F4 (HF5) [[5], and references therein]. Most recent applications of F4 span from protein to nanoparticle [6–14]. F4 is universally applied to separate macromolecular solutions and particle suspensions based on differences in diffusion coefficient and, consequently, on hydrodynamic size or molar mass within a broad dynamic range (1 nm–50 μm) [3]. Both soluble macromolecules and particulates can be analyzed in one experiment with high resolution (key feature when "free" reagents have to be separated from the fraction that is actually "bound" to functional particles [15]). Because separation takes place without the use of a stationary phase as applied in column chromatography, there is less danger of sample adsorption or physical plugging of the separation channel.

Among the F4 variants, HF5 is the only one that has up to now not been commercially available. HF5 uses a completely different channel geometry based on a polymeric or ceramic hollow-fiber with porous walls as a cylindrical channel.

When a flow is introduced into the channel, it will partly permeate the walls and create a radial cross-flow whereas the remainder will exit the fiber, carrying the sample fractions towards the detector.

Although HF5 has been utilized only by few research groups, the literature shows promising results for protein, nanoparticles, and even whole cell fractionation [17–20]. Very interesting and unique features of HF5 motivate development of this technique for applications in emerging bioanalytical fields such as protein analysis and proteomics, particularly when coupled with mass spectrometry [21–24]. The hollow fibers that have been mostly used in prototype HF5 channels are readily available from manufacturers of water purification cartridges; they are low-cost material, and consequently, allow the construction of a fractionation channel which

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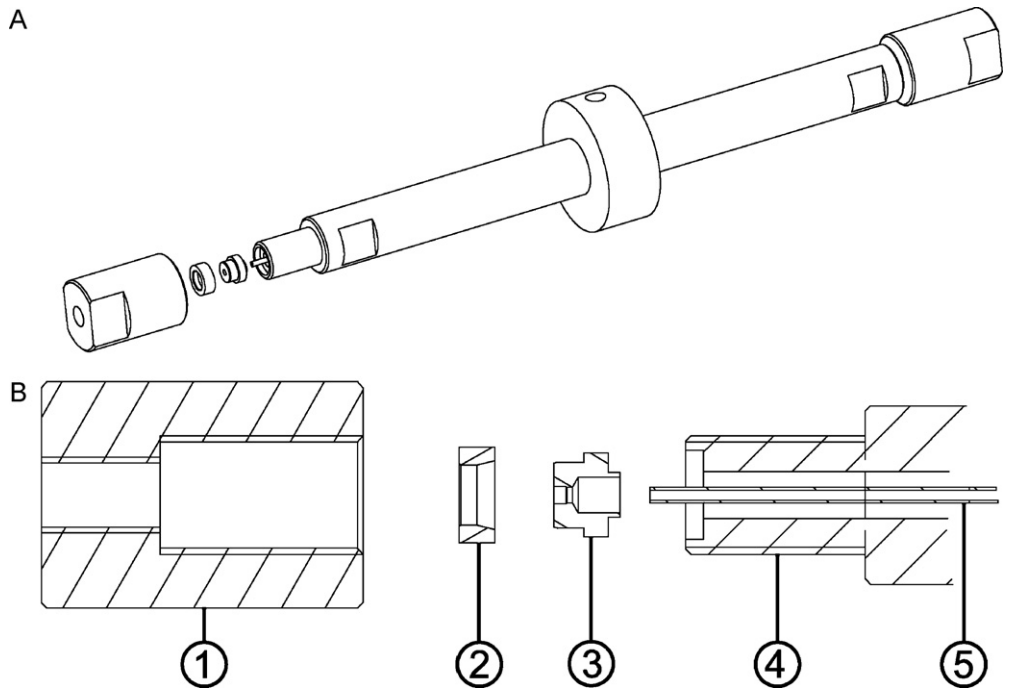

Fig. 1. Scheme of the HF5 cartridge design. It is an assembly (overview A) which consists (exploded view B) of a tube housing of the hollow fiber, with length of 17 cm, the hollow fiber itself (5), two cap nuts (1), two gaskets (3), and two stainless steel ferrules (2) with a inner chamfer on one site. The hollow fiber is sealed with the stainless steel ferrule and the gasket. By tightening the cap nut, the ferrule and the gasket compress the hollow fiber to create a seal against the volume outside the hollow fiber, ensuring that the flow and the sample are introduced into the hollow fiber.

is disposable. A new, fresh membrane could be introduced even after a single run, which is a key feature to avoid run-to-run sample carryover and sterility issues. The low channel volume of typically less than 100 μl reduces flow rates and sample dilution.

To overcome the limitation known from the literature of a tedious manual assembly of the HF5 channel and, even more serious, inferior separation efficiency [25] was the goal of our work.

Another scope of improvement relates to a specific characteristic of the AF4 and HF5 separation mechanism. With these F4 methods the cross-flow is generated from the channel flow, in contrast to symmetrical F4. A so called "focusing/relaxation" step is used, during which flow is introduced from both ends to the channel requiring the flow rate ratio to be adjusted so that the flows meet downstream of the inlet port. Typically one aims at a ratio of 1–9 that is a 9-fold flow rate entering from the outlet port of the channel. The flow profiles at the focusing point are directed perpendicular towards the porous membrane. The sample is injected and transported into the channel through the flow entering the inlet port. Once the sample components have reached the focusing zone, they will be concentrated in a narrow band and at the same time they are exposed to the cross-flow and relaxation is achieved after some time. The correct position of the focusing flow is a critical parameter for a successful separation. Up to now it is commonly verified by injecting a colored sample (e.g. dextrane blue) and observing the focus band through a transparent channel cover plate. In HF5 where the channel cannot be made transparent, visualization is not feasible and flow rates are measured by removing the channel. It cannot be excluded that the effective flow rates during focusing are different or that they change with ageing of the membrane. The improvements suggested in this work provide better control of the focusing conditions, making it easy to place the focus zone at any desired place in the channel and to verify this position in real time for every experiment.

Sample concentration during focusing/relaxation may cause unwanted aggregation or association to higher order species [16]. The developments presented here allow influencing the width of the focusing zone. A wider zone helps to reduce sample concentration and therefore minimizes overloading and sample interactions.

Our aim is to convince more researchers to use F4 instrumentation and prepare the ground for a breakthrough in terms of achieving a significant user base compared to other size-based separation methods, like size exclusion chromatography (SEC). Complexity of F4 instrumentation needs to be reduced and maintenance of F4 channels greatly simplified in order to achieve this goal. The effort to run an F4 experiment should not exceed the skills required for HPLC or SEC.

Here we present for the first time a new F4 system suitable to be used with both AF4 and HF5 channels. The new system equipped with either a two-port AF4 channel or an HF5 cartridge, provides the flexibility to change from AF4 to HF5 mode in the same instrumentation. It is shown that the new design improves the separation efficiency and reproducibility of HF5.

2. Experimental

2.1. HF5 channel design

The HF5 channel reported in Fig. 1 is a construction consisting of a tube housing of the hollow fiber, with length of 17 cm, the hollow fiber itself, two cap nuts, two gaskets, and two stainless steel ferrules with an inner chamfer on one side. The hollow fiber is sealed with the stainless steel ferrule and the gasket. By tightening the cap nut, the ferrule and the gasket compress the hollow fiber to form a seal against the outer volume, ensuring that the flow and the sample are introduced into the fiber.

The cartridge is sealed up to 30 bar (435 psi).

The hollow-fiber material used in the cartridge was polyethersulfone with a nominal molecular weight cutoff of 10 kDa, which corresponds to an average pore size of 5 nm according to the manufacturer; 0.8 mm ID, and 1.3 mm OD (Fiber type FUS 0181, Microdyn-Nadir, Wiesbaden, Germany).

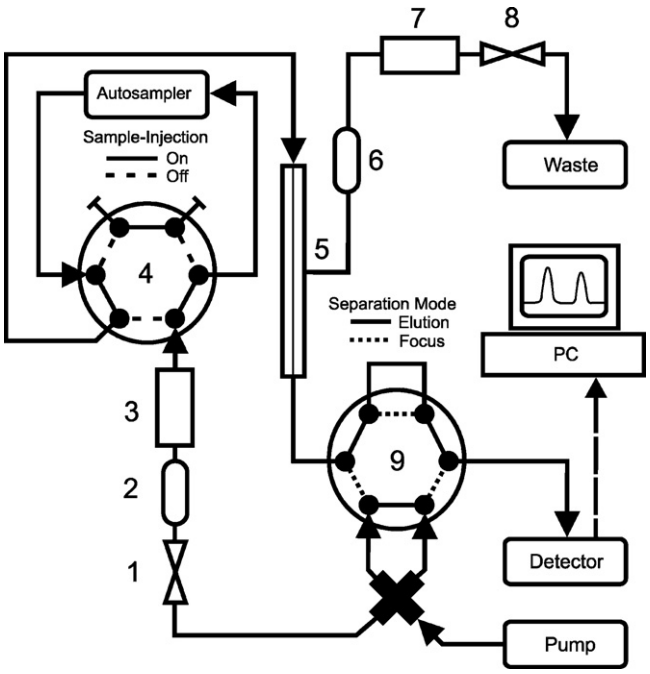

Fig. 2. Flow schematic of the novel flow control module that can operate either in AF4 or in HF5 mode. The system consists of the following elements: (1) metering valve, (2) pressure sensor, (3) flow measuring device, (4) six port switching valve, (5) HF5 cartridge or AF4 channel, (6) pressure sensor, (7) flow measuring device, (8) metering valve, and (9) six port switching valve.

2.2. Two-port AF4 channel

One of the features of current F4 instrumentation which leads to complications for switching between AF4 and HF5 (or packed column in the same system) is the number of fluid connections of the separation device. AF4 channels use three fluid connections in the upper plate [26] (inlet, injection and outlet), while HF5 channels have only two, like chromatography columns. Therefore, the AF4 channel was newly designed with only two fluid connections in the cover plate for the mobile phase inlet and outlet ports, with sample injection then made through the mobile phase inlet port. Channel height was 490 μm, and a regenerated cellulose membrane with cutoff 10 kDa was used (Microdyn-Nadir, Wiesbaden). The channel had a length of 171 mm, a width at focusing zone of 21.5 mm.

2.3. F4 system

In Fig. 2 a scheme of the new AF4 system able to operate either with a two-port AF4 channel or with an HF5 channel is presented. As mentioned before, F4 requires several flow rates that need to be controlled independent of each other with high precision and accuracy. The system shown in Fig. 2 creates all necessary flows using one pump only, whose flow is split in a controlled and dynamic way. To achieve this, the pump is connected to a cross piece. In focus mode three different flows are created. One is a bypass flow through the detector, which prevents signal perturbations at the start of the elution phase. The second flow leads to the inlet port of the separation channel (5). The flow meter (3) (miniCoriFlow, Bronkhorst HighTec, Ruurlo, Netherlands) measures the flow rate in real time and the output is used to adjust the metering valve (1) to reach the desired flow rate. The third flow rate results automatically as the difference to the pump flow rate. The six-port valve (4) is used to enable the injection mode of the autosampler. In this way it is possible to control the focus position in a precise and accurate way. The ratio of the two focusing flow rates can be adjusted and this will place the focusing zone at a position given by the same ratio of the distance from the inlet and outlet port. This procedure replaces an empirical balancing of flow rates by injection of a colored sample (usually dextrane blue). It does not require a transparent upper channel plate. In case of an HF5 channel, the ratio can be adjusted for each new fiber installed; even for each focusing process individually. The metering valve is motorized and under software control it can be moved continuously during focusing. This spreads the focusing band to a wider area, reducing sample concentration.

The inflow to the channel exits as cross-flow and is measured and regulated with a flow controller (7/8) (miniCoriFlow Controller, Bronkhorst HighTec, Ruurlo, Netherlands).

In elution mode, the six-port valve (9) is switched leading to a different flow pattern. The pump flow is leading without any split to the channel inlet. The flow controller (7/8) adjusts the cross-flow to the value required by the separation method. Variations of the cross-flow rate can be readily generated.

Two pressure sensors are installed, one (2) monitors the hydrostatic pressure in the channel (there is no pressure drop from its position to the channel interior), the other one (6) is placed beneath the membrane. The difference of the two pressure readings is equivalent to the pressure drop across the membrane. This is a useful parameter to indicate the state of the membrane.

The HPLC instruments were from Agilent Technologies (Santa Clara, USA) with a 1100 Agilent degasser, a 1100 Agilent isocratic pump, a 1200 Agilent auto sampler, and a 1100 Agilent variable wavelength detector. A DAWN HELEOS II 18-angle light scattering detector, and an Optilab rEX refractive index detector (Wyatt Technology Corporation, Santa Barbara, USA) were also employed.

2.4. Samples and chemicals

Samples were solutions of carbonic anhydrase (30 kDa), bovine serum albumin (BSA, 66 kDa), apoferritin (481 kDa), and thyroglobulin (670 kDa) (Sigma, St. Louis, USA) in the carrier solution. Carrier solutions were prepared in water purified by an Elix 3 UV Water Purification System (Millipore, Billerica, USA), and filtered through 0.1 μm-pore membrane filter.

Two different carrier solutions were used: (1) 50 mM $NaNO_3$ for BSA measurements, (2) 10 mM PBS + 150 mM NaCl for the mixture of the four proteins. The injection amounts were 5 μl of a 1 mg/ml BSA solution, and for the protein mixture 5 μl of a solution with 1 mg/ml of each protein, resulting in a total concentration of 4 mg/ml.

2.5. AF4 and HF5 flow rate conditions

In AF4, the fractionation of BSA was performed with a longitudinal flow rate of 1.00 ml/min and a cross-flow rate of 3.00 ml/min. Before the elution step the sample was focused for 2 min at a cross-flow rate of 3 ml/min. The same experimental conditions were applied also for the analysis of the mixture of proteins, but in this case the cross-flow was linearly reduced from 3.00 ml/min to 0.00 ml/min in 16 min, starting after 14 min run time. Start of elution was at 4 min run time, because a 2 min equilibration phase in elution mode was done before starting the focusing/injection step.

In HF5, the fractionation of BSA samples was performed with a longitudinal flow rate of 0.35 ml/min. The focusing step was performed for 4 min with a focusing flow rate of 0.85 ml/min. During the elution, the cross-flow was maintained constant at 0.85 ml/min until the complete elution of sample components. For the mixture of proteins, the detector flow was set at 0.2 ml/min and the cross-flow at 0.5 ml/min. The cross-flow was maintained constant for 14 min and then reduced to 0.00 ml/min in 16 min in a linear manner.

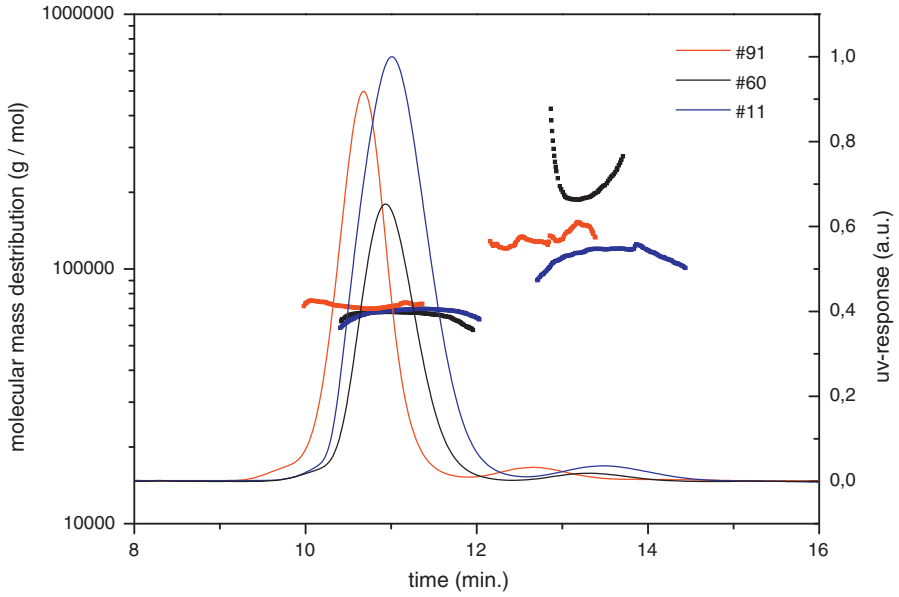

Fig. 3. Overlay of three fractograms chosen from a sequence of 100 HF5 runs: run #11 in blue, run #60 in black, and run #91 in red. Sample is BSA, the experimental conditions are given in Sections 2.4 and 2.5. The UV signal was recorded at 280 nm. MALS signals were used to determine the molar mass values. The fractograms are normalized to the same height. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3. Results and discussion

Separation efficiency and run-to-run reproducibility of HF5 was tested by performing a sequence of 100 BSA runs on the same channel and fiber. Fig. 3 shows the fractograms obtained from the 11th (blue trace), 60th (black trace) and 91st (red trace) run. Molar masses of the eluted fractions were calculated from the light scattering signals at the retention time maxima, and they are also shown in Fig. 3. The values measured were close to the expected values of 67 kDa. In Table 1 the relevant values of retention time, resolution between monomer and dimer, plate height, and symmetry values of the BSA monomer peak are listed. There is a slight, but distinct trend to more narrow peaks and higher resolution with increasing number of injections. Currently we cannot explain this effect. However, the results indicate higher efficiency and resolution compared to what is reported in the HF5 literature [17]. Recovery, which is also given in Table 1 is lower than expected with 60% average. The low recovery is sample dependent, because for the protein mixture a recovery of 90% was found. We cannot yet explain the reason for the sample loss, it could be caused by permeation through the membrane wall of the fiber, although the cut-off is specified to be 10 kDa.

Remarkably, performance in terms of resolution and peak shape is comparable to what is obtained in AF4 mode, as can it can be seen in Fig. 4. Because the BSA eluted later on the AF4 channel its elution time axis was shifted by 0.65 min to align the left peak flanks for better comparison of peak shape and width. The UV signal was chosen, because it has the lowest secondary band broadening of all detectors. The figure illustrates that in principle similar resolution and efficiency can be achieved with an HF5 channel. This is confirmed by comparing plate numbers and resolution for both fractograms. For the AF4 case in this specific example the plate number is 480 and resolution 1.45 versus 560 and 1.53 for HF5 (run #91 from Table 1). Work is in progress to confirm these results and to further analyse the separation properties of HF5 compared to AF4.

To confirm increased resolution and efficiency also within a broader molar mass range, the mixture of four proteins was run on the HF5 channel. Fig. 5 reports the fractograms (solid blue line is the RI trace) with molar masses calculated from light scattering (solid red line). The cross-flow rate is shown in a dashed blue line. Molar mass determination is reasonably accurate even for a mix of proteins whose components are not completely baseline separated. The results show that it is possible to use the RI signal for reliable concentration determination. This is important, because for protein

Table 1
Retention time (tr), relative retention between monomer and dimer (dtr), resolution (R), peak symmetry for the monomer, peak width of the monomer at half height, plate height (H) and recovery (MR) values for a series of 100 injections of BSA.

Run	tr 1 monomer (min)	dtr (min)	R	Symm. monomer	Width (min)	H (cm)	MR (%)
11	11.05	2.49	1.47	1.04	0.8676	0.046	91.7
20	10.83	2.42	1.69	0.9	0.7451	0.037	67.8
31	10.86	2.41	1.58	0.89	0.701	0.032	51.4
40	10.99	2.51	1.65	0.86	0.6863	0.030	52.7
51	11.06	2.35	1.67	0.88	0.6765	0.028	47
60	10.96	2.41	1.75	0.85	0.6569	0.027	48.2
71	10.93	2.36	1.74	0.87	0.6593	0.028	47.9
80	10.9	2.14	1.59	1.08	0.6029	0.023	61.6
91	10.72	1.92	1.53	1.25	0.576	0.023	61.3
100	10.56	1.86	1.59	1.34	0.5564	0.022	62.9
Average	10.89	2.29	1.63	1.00	0.67	0.03	59.25
SD	0.1539	0.2326	0.0902	0.1768	0.0897	0.0074	13.5592
RSD (%)	1.41	10.17	5.55	17.75	13.33	25.02	22.88

Experimental conditions as given in Sections 2.4 and 2.5.

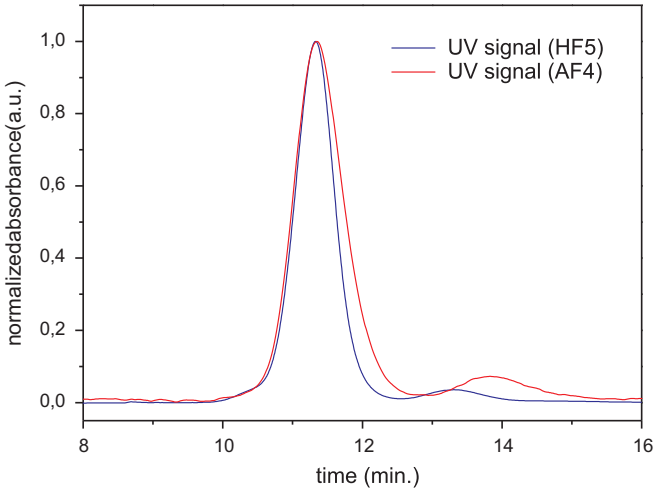

Fig. 4. Comparison between HF5 (run #91 from Table 1; in blue) and AF4 (in red) fractograms of BSA. Experimental conditions are given in Sections 2.4 and 2.5. UV signal recorded at 280 nm. The fractograms are normalized to the same height of the monomer peak, and because the BSA eluted later on the AF4 channel, its elution time axis has been shifted by 0.65 min to align the left peak flank for better comparison of the peak shape and width. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

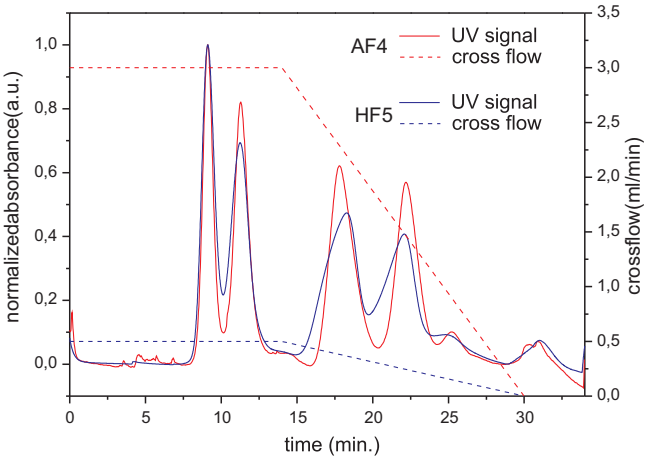

Fig. 6. Comparison between HF5 (in blue solid line) and AF4 (in red solid line) UV fractograms of the same protein mixture as shown in Fig. 5. Cross-flow rates are shown as dotted line for HF5 and AF4 in the same colour. Experimental conditions are given in Sections 2.4 and 2.5. UV signal recorded at 280 nm. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

mixtures which are not baseline-separated, concentration cannot be extracted from the UV signal at 280 nm.

Separation quality is superior to what was previously reported by other authors [17]. Run-to-run reproducibility was also high (data not shown). A comparison of the separation with HF5 versus AF4 is shown in Fig. 6. Both traces (AF4 red, HF5 blue) are plotted on the same retention time axis, the separation methods were adjusted to create a perfect overlay of the retention time of all four proteins. The cross-flow rates as a function of retention time are also shown with a dotted line. For the two lowest molar mass proteins, resolution and efficiency were also comparable to what was obtained with the AF4 channel. Nevertheless, the two highest molar mass proteins show asymmetrical HF5 peaks. The reasons for this behaviour, however, need to be investigated in further studies.

4. Conclusions

The results show that using the new system design, the gap between HF5 and AF4 is closed with respect to separation efficiency.

The new F4 system proposed here differs in the following ways from the schemes previously described in the literature:

1. only one pump is used;
2. the cross-flow is not generated by a pump working in aspiration mode but it is regulated with a flow meter device;

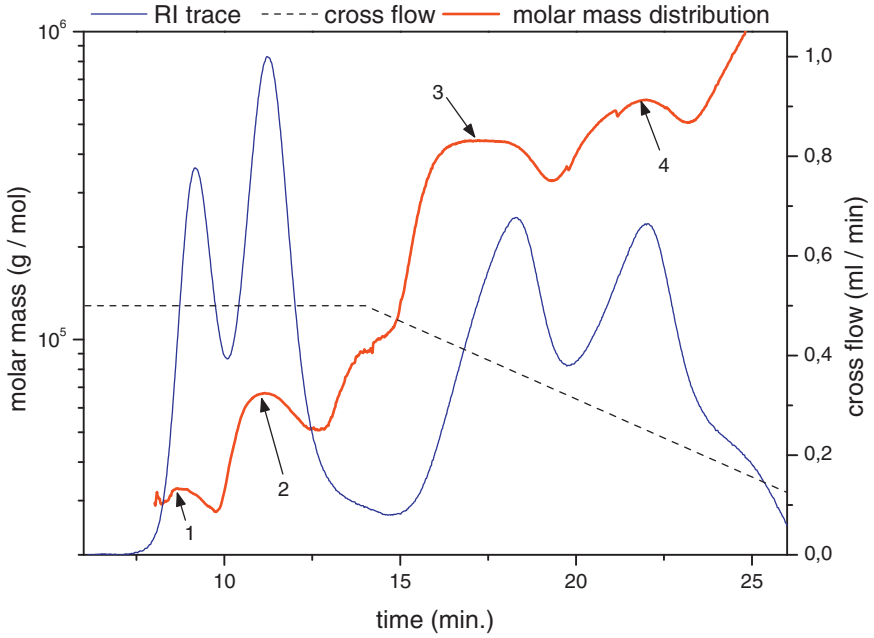

Fig. 5. HF5 separation of the protein mixture carbonic anhydrase (1), BSA (2), apoferritin (3), and thyroglobulin (4). RI fractogram (in blue solid line against arbitrary units) and calculated molar mass values determined by MALS signals (in red). Plateau values and percent standard deviation: (1) 31.3 kDa (15%); (2) 67.1 kDa (5%); (3) 447 kDa (1%); (4) 615 kDa (8%). Experimental conditions are given in Sections 2.4 and 2.5. Cross-flow rates are shown as dotted line. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

3. the focus position is determined and regulated in a novel way that avoids possible shifts of the position away from the chosen, optimal point, and it also allows to dynamically adjust and shift the focusing position;
4. the HF5 cartridge is built using a novel sealing mechanism. All mechanical parts can be manufactured in large quantities. Installing the fiber into a channel cartridge is straightforward. Thus an access to an unlimited number of HF5 channels can be provided. One cartridge is replaced with another in a few seconds by connecting three pieces of tubing with finger tight fittings;
5. the AF4 channel has two connecting ports in the upper pate, making it easier to use it together with HF5.

At the current stage of development, we are confident that the new F4 system offers higher potential to F4 users because of the enhanced HF5 performance, and of flexibility to alternatively use AF4 or HF5 channels.

Conflict of interests

Wyatt Technology Europe GmbH is a company that produces and commercializes F4 systems. Novel aspects of F4 system and HF5 channel design have been filed for patent. Mission of the academic spinoff company byFlow Srl includes know-how transfer, development, and application of novel flow-assisted technologies and methodologies for the analysis and characterization of samples of nano-biotechnological interest.

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