Supplementary Figures

FlashPack: Fast and simple preparation of ultra-high performance capillary columns for LC-MS

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Supplementary Fig. S1

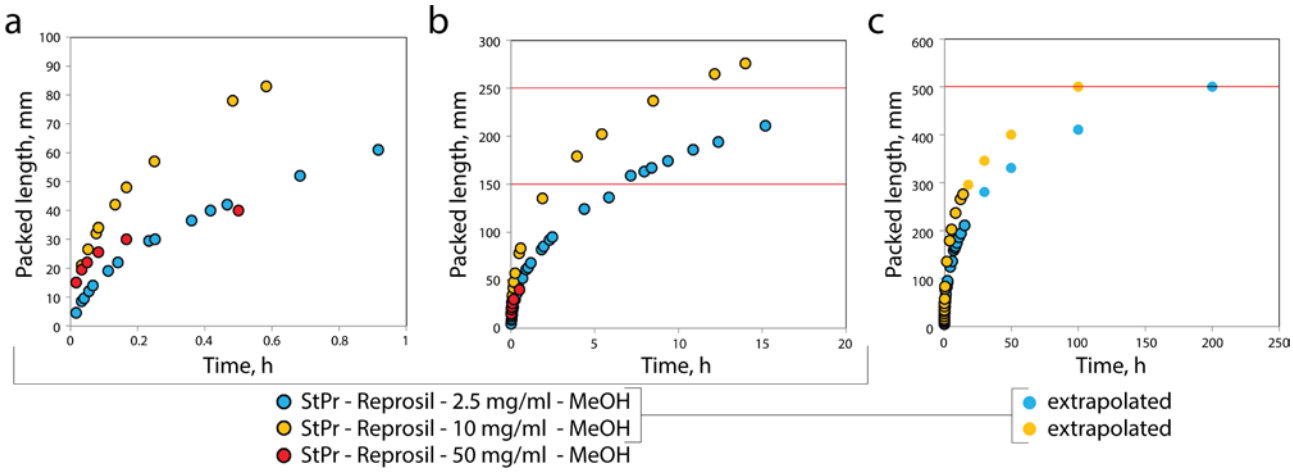


Fig. S1 Sorbent suspension concentration dependence of the column packing rate and length.

(**a**) Packing rate increases when sorbent suspension concentration is raised from 2.5 to 10 mg/ml. Further sorbent concentration increase to 50 mg/ml leads to dramatic reduction in the packing rate. **(b)** At 100 bar pressure packing of 15 cm columns requires from 3 to 7 h, packing of 25 cm columns takes from 10 to 25 hours depending on the sorbent concentrations (2.5 or 10 mg/ml respectively). **(c)** Extrapolation of the packing rate indicates that packing of 50 cm columns would require from 4 to 8 days.

Supplementary Fig. S2

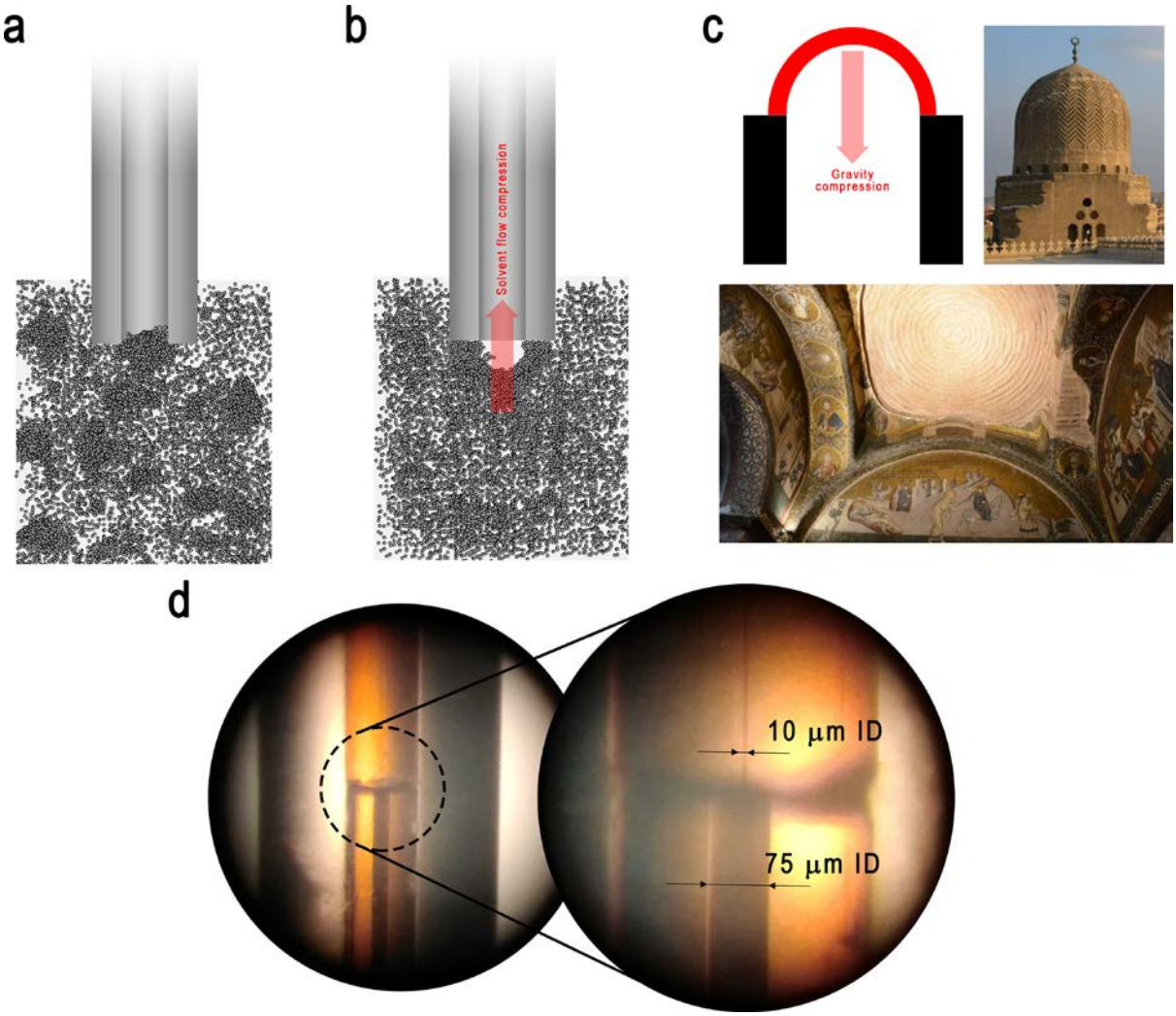


Fig. S2 Column entrance blocking at high sorbent concentration.

(a) Irreversible clogging and blocking of the capillary column entrance by large sorbent particle aggregates. **(b)** Capillary blocking by a dynamic self-assembling structure "cupola", formed by sorbent particles aggregating around the column entrance. The structure probably resembles that of a brick cupola dome in architecture. **(c)** The domes of Faraq Ibn Barquq mosque, Cairo, Egypt and Church of the Holy Savior, Istanbul, Turkey. In such cupola/dome-like structure the sorbent particles can be regarded as bricks, their interaction and aggregation as mortar and the solvent flow provides an equivalent of gravity and compression to stabilize the cupola. **(d)** A similar self-assembling stable "cupola" structure likely forms between capillaries of different internal diameters. Here 75 μm ID capillary is mounted in the pressure bomb, while its distant end is connected to 10 μm ID capillary. When the bomb is pressurized the 75 μm ID capillary is packed with sorbent while the 10 μm ID capillary stays empty.

Supplementary Fig. S3

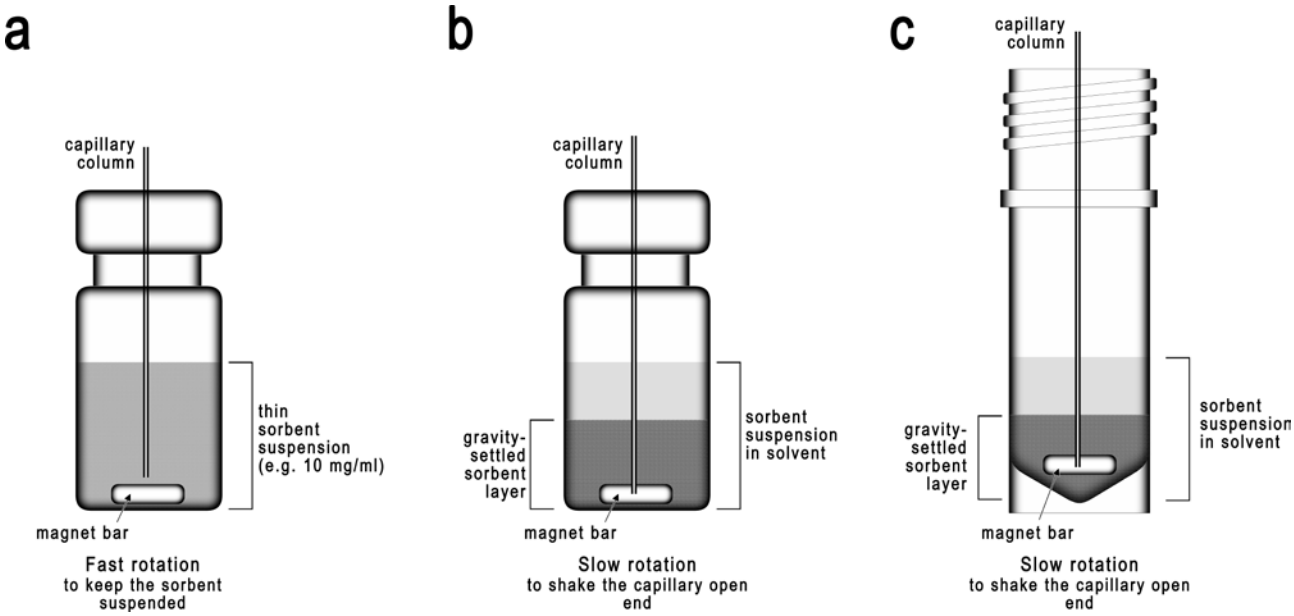


Fig. S3 Sorbent vials and capillary positioning.

The standard column packing approach **(a)** uses slurry of sorbent at low concentration, which is kept in suspension by a fast-rotating magnet bar. The FlashPack approach **(b)** uses a very high sorbent concentration in the form of a gravity-settled sorbent layer. The rotating magnet bar is used to tap the proximal capillary end. The magnet rotates at a minimum speed (300-400 rpm) to destabilize the cupola structure whilst avoiding sorbent layer resuspension. The FlashPack approach uses a relatively large amount/volume of the sorbent, which can be reduced by using of conical-bottom vial **(c).**

Supplementary Fig. S4

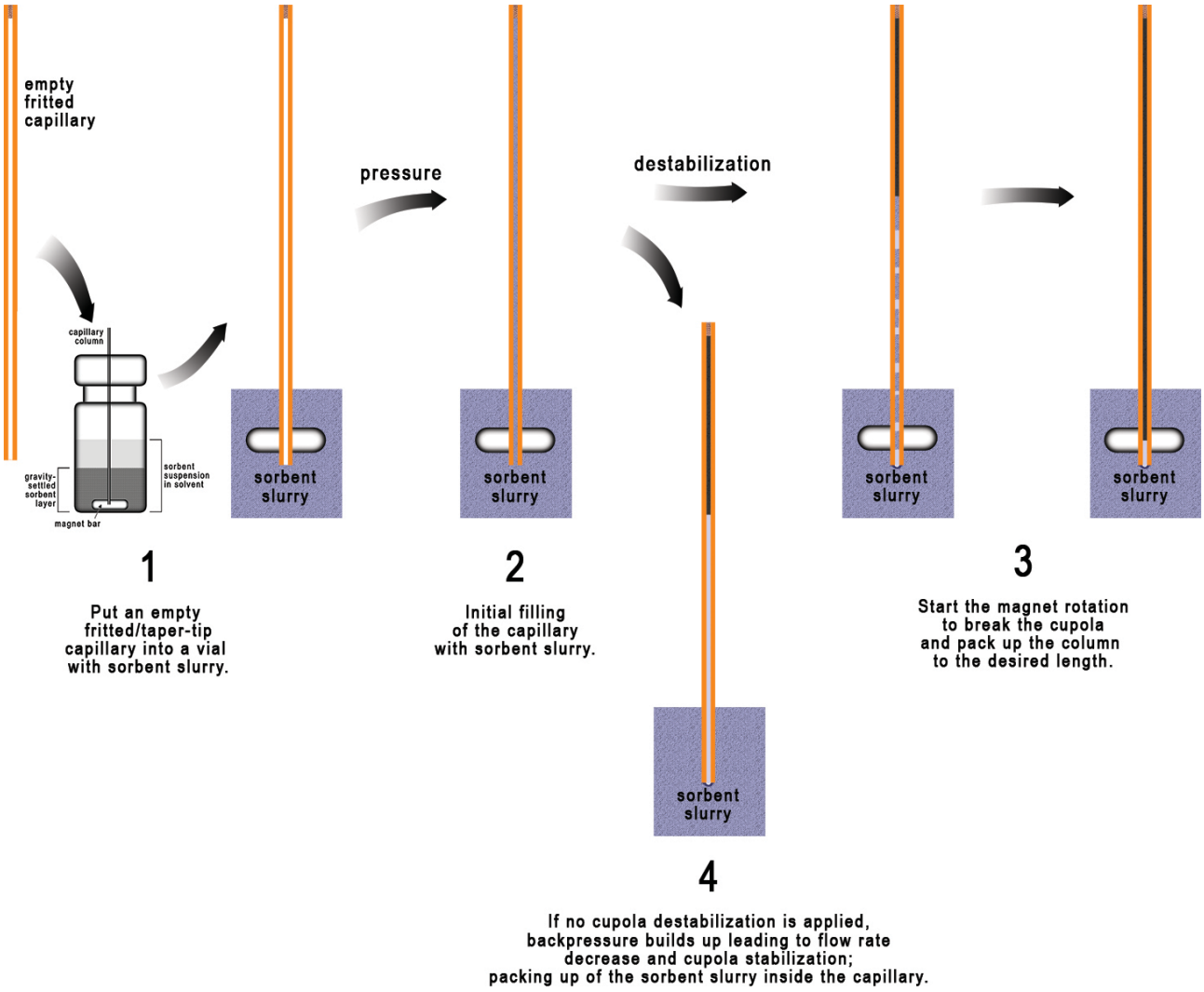


Fig. S4 FlashPack packing process

(1) The column is mounted inside the sorbent vial. **(2)** The system is pressurized and the sorbent starts to enter the capillary. **(3)** Magnet-bar assisted cupola destabilization prevents column entrance blocking and allows for uninterrupted packing to the desired length. **(4)** Without destabilization, the solvent flow packs the sorbent material which entered the column during initial filling on step (2). The cupola block prevents new material to enter. For additional details please see results and discussion section.

Supplementary Fig. S5

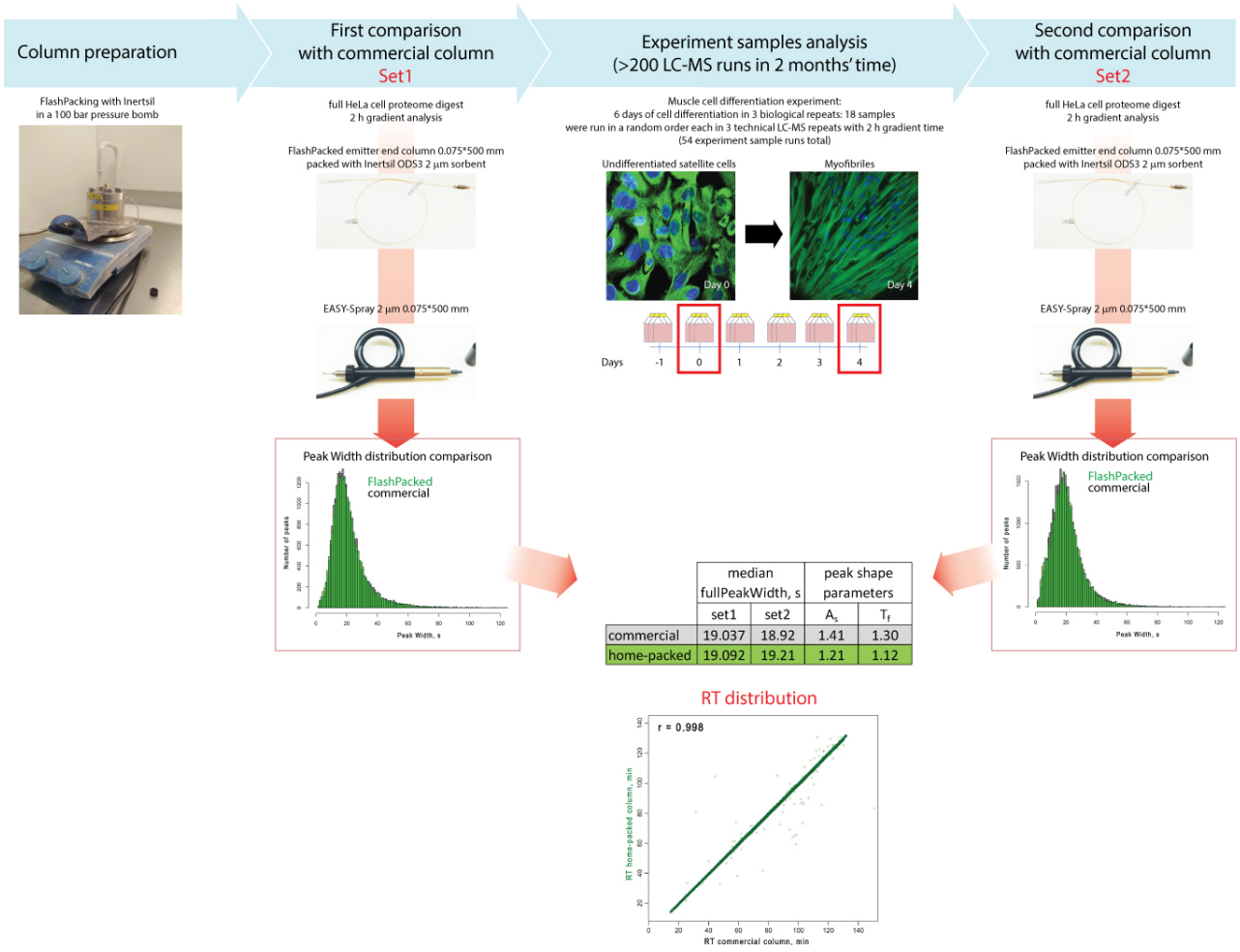
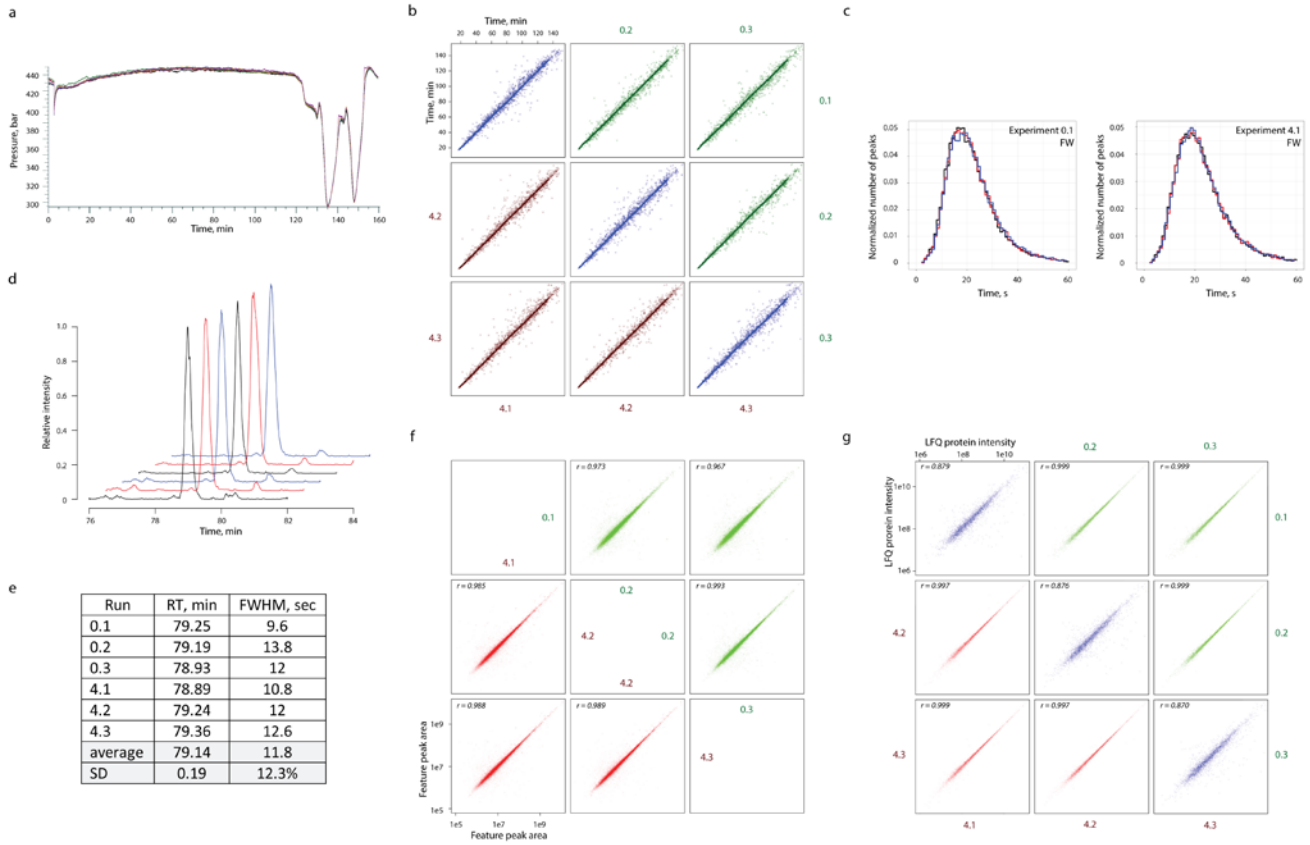


Fig. S5 FlashPack column durability and performance test

The FlashPack column was used during a 2 months period for more than 200 LC-MS runs. Experimental samples included a set of 18 muscle cell differentiation samples analyzed continuously in 54 * 2 h gradient runs in 2 weeks. The results for triplicate technical repeats for samples from Day 0 and Day 4 of differentiation were used for technical reproducibility and biological feasibility analysis (Supplementary Fig. S6). Column performance was tested before and after experimental samples using a HeLa protein extract (tryptic digest) and compared to a commercial capillary column.

Supplementary Fig. S6



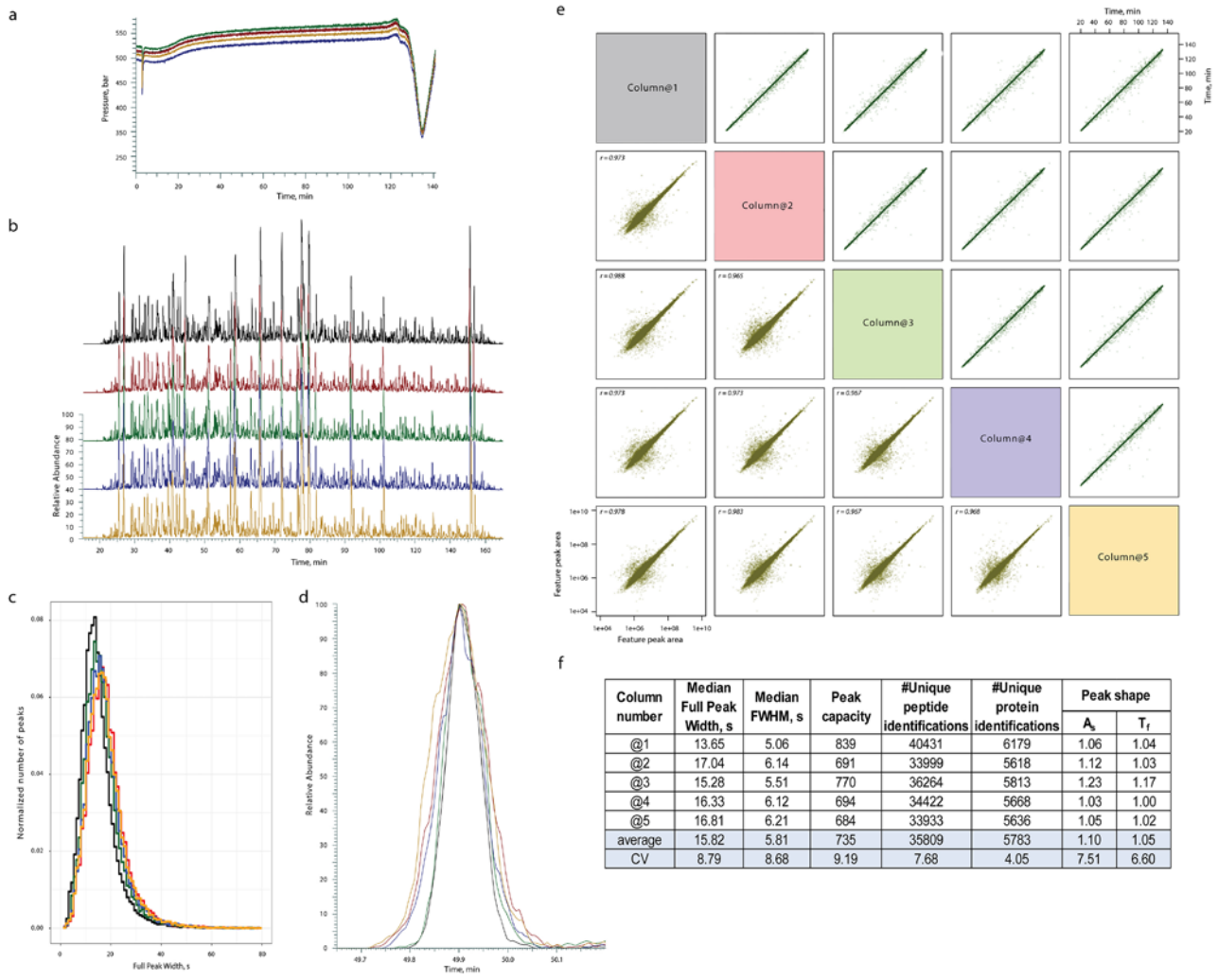
Run	RT, min	FWHM, sec
0.1	79.25	9.6
0.2	79.19	13.8
0.3	78.93	12
4.1	78.89	10.8
4.2	79.24	12
4.3	79.36	12.6
average	79.14	11.8
SD	0.19	12.3%

Run	median FullPeakWidth, sec	median FWHM, sec	Sample peak capacity	# of unique Peptide Identifications	# of unique Protein Identifications
0.1	19.12	6.68	636	26136	4049
0.2	19.33	6.67	637	25980	4062
0.3	19.46	6.7	634	25868	4054
average	19.30	6.68	636	25995	4055
CV	0.89	0.23	0.23	0.52	0.16
4.1	19.84	6.89	617	25684	3825
4.2	19.93	6.97	609	25602	3811
4.3	19.9	7.03	604	25529	3843
average	19.89	6.96	610	25605	3826
CV	0.23	1.01	1.01	0.30	0.42

Fig. S6. Reproducibility and performance of a single FlashPack column (50 cm, 75 um ID, Inertsil ODS-3 2 µm sorbent) during quantitative proteomics analysis of muscle cell differentiation (day 0 and 4).

Protein extracts from myocytes harvested at day 0 and 4 of differentiation were digested and analyzed in a 2 h LC-MS gradient in triplicates (runs 0.1 - 0.3 and 4.1 - 4.3). The column demonstrated a high level of robustness as seen from (**a**) pressure and (**b-e**) stable separation characteristics over time: both by generalized correlations between (**b**) peptide retention times (Pearson correlation r>0.997 for all pairs of LC-MS analyses) and (**c**) peak width distributions. Individual peptide performance was very good: the extracted ion chromatograms (XICs) of chromatographic feature (m/z 540.2846, z = +2) were generated for six runs (**d**) and corresponding FWHM and RT parameters were analyzed (**e**). Separation reproducibility translates into very high quantitative reproducibility between technical repeats of LC-MS experiments on both (**f**) peptide and (**g**) protein levels. The results on separation and identification reproducibility are summarized in the table (**h**). The biological compliance of the data is proved by the analysis of 4 markers of muscle cell differentiation (**i**). Fold changes (FC) were calculated based on MaxQuant LFQ intensity values normalized against average $\log_2$(LFQ intensity) for sample 0.

Supplementary Fig. S7



Column number	Median Full Peak Width, s	Median FWHM, s	Peak capacity	#Unique peptide identifications	#Unique protein identifications	Peak shape	
						A_s	T_f
@1	13.65	5.06	839	40431	6179	1.06	1.04
@2	17.04	6.14	691	33999	5618	1.12	1.03
@3	15.28	5.51	770	36264	5813	1.23	1.17
@4	16.33	6.12	694	34422	5668	1.03	1.00
@5	16.81	6.21	684	33933	5636	1.05	1.02
average	15.82	5.81	735	35809	5783	1.10	1.05
CV	8.79	8.68	9.19	7.68	4.05	7.51	6.60

Fig. S7 FlashPack column packing reproducibility and performance.

Five FlashPack columns (70cm, 75 μm ID, Inertsil ODS-3 2 μm) were tested using HeLa cell tryptic digest and 2h LC-MS analysis. The results for columns from 1 to 5 are shown in black, red, green, blue and orange colors respectively**.** Packing reproducibility and robustness is demonstrated by (**a**) very similar back pressure traces (497 to 523 bar at 250 nl/min for 2% buffer B at 55^{o}C) and (**b**-**f**) separation characteristics of independently packed columns. The columns produce (**b**) almost identical base peak current chromatographic profiles with (**c**) very similar peak resolution characteristics (baseline peak width distribution), (**d**) good peak shape with just minor tailing (XICs for an example chromatographic feature with m/z 594.33, z = +2 are shown with retention time (RT) aligned to allow the overlay) and (**e**) excellent RT reproducibility (dark green traces, no alignment, Pearson's correlation r>0.995 for all pairs). High column packing reproducibility translates into (**e**) high quantitative LC-MS reproducibility (feature peak areas are shown in light-green traces, Pearson's correlation is calculated for each plot); (**g**) table summarizing columns performance characteristics.