List of Supporting Information:

- **Table S1 (see p. S-4): Identified proteins**

List of the mouse proteins identified by SAX-RP, RP-RP and combined search.

- **Figure S1 (p. S-5): Evaluation of two C18 RP columns for 2D RP/RP LC**

The 2D retention maps of 6-mix protein digests. The first dimension was either performed on an Acclaim 120 (classical C18, left) or Acclaim Polar Advantage (PA2, right) reversed-phase columns (both Thermo-Fisher Scientific). The second dimension was performed on a Gemini C18 (Phenomenex). The polar-embedded Acclaim PA2 performed significantly better in orthogonality and in efficiency and was therefore chosen for comparison with the AS24 hSAX column.

- **Figure S2 (p. S-6): Chromatography analysis of 6-mix and total cell lysate digests on the IonPac AS25**

The AS25 has similar backbone chemistry as AS24 and is the most hydrophilic member of the IonPac family. Tryptic digests of 6-mix (left) and RAW264.7 cell lysate (right) were analysed on the AS25 confirming that hydrophilicity of the backbone plays a key role in the separation power of strong anion exchange fractionation. As AS25 shows lower resolution than AS24, the latter was chosen for further experiments.

- **Figure S3 (p. S-7): Performance of peptide separation by AS24 at different pH**

(A) Separation of tryptic peptides from 6mix was evaluated when the sample, after desalting, was re-suspended either in 20 mM Tris (pH 8) or in de-ionized water (pH 4). Sample loading at pH 8 resulted in better separation than pH 4. This is additionally beneficial as tryptic digests performed in 20 mM Tris-HCl, pH 8 can directly be injected onto the column, minimising sample loss.

(B) Complex mixtures of tryptic peptides from RAW264.7 macrophage cell lysate were analysed at pH 8.0 (20mM Tris-HCl) and pH 10 (20mM Tris-HCl + NH4OH). As expected, the orthogonality was conserved at higher pH but the resolution was slightly compromised.

- **Figure S4 (p. S-8): AS24 column efficiency**

The chromatogram shows the separation of tryptic digested BSA (140µg).
The table reports the averaged values of the parameters described below, which are used to define the column efficiency.

- **Figure S5 (p. S-9): Reproducibility of the AS24 SAX column**

(A) UV chromatogram of three replicate injections of 100 µg of tryptic digests of 6-protein mix ("6-mix") on the AS24 SAX column. **(B)** q-q plot of peak areas from fractions of the same experiment as in (A) run in a 2D SAX/RP approach show very high reproducibility.

- **Figure S6 (p. S-10): Linearity of AS24 SAX column**

Analysis of the linear response of AS24 SAX column shows a very good reproducibility and linearity over almost an order of magnitude (30-240 µg sample loading) in a UV chromatogram of the first dimension **(A)** and in a q-q plot of peak areas of randomly selected peaks from fractions run in a 2D SAX/RP approach **(B)**.

- **Figure S7 (p. S-11-12): Selected base peak chromatograms of SAX and RP fractions analysed by LC/MS**

Base peak chromatograms of the online RP LC of SAX and high pH RP fractions show a high degree of orthogonality comparable to the offline 2D fractionation.

- **Figure S8 (p. S-13): Sequence coverage of identified proteins in SAX/RP and RP/RP approaches**

Both hSAX/RP and RP/RP approaches showed similar sequence coverage of proteins identified with an average of 31% and 32%, respectively. However, hSAX identified more proteins with higher sequence coverage.

- **Figure S9 (p. S-14): Characteristics of peptides identified uniquely in hSAX/RP or RP/RP approaches**

Peptides unique to the RP approach (blue), the hSAX approach (red) and peptides shared (green) were analysed for their individual content of amino acid groups. The data shows that the peptides unique to hSAX and RP are similar for the content of most amino acids except for acidic amino acids Glutamate (E) and Aspartate (D), where hSAX favours the identification of more acidic peptides.

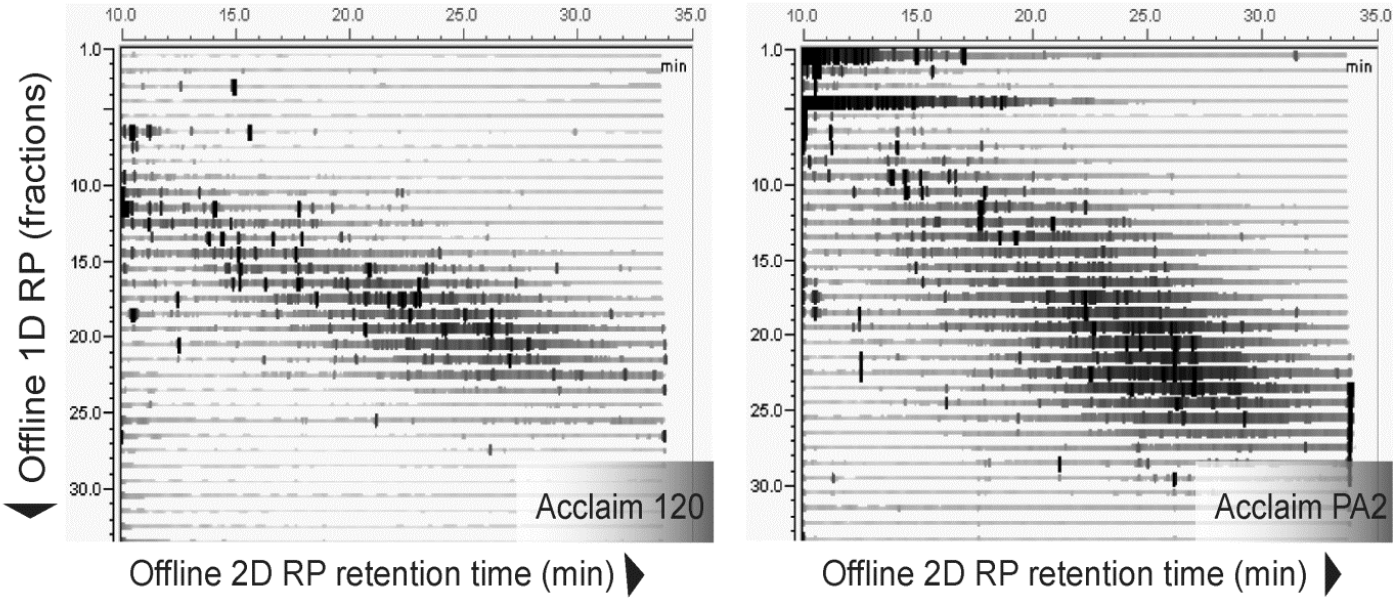
Supporting Information, Table S1

See the Supporting Information, Excel files:

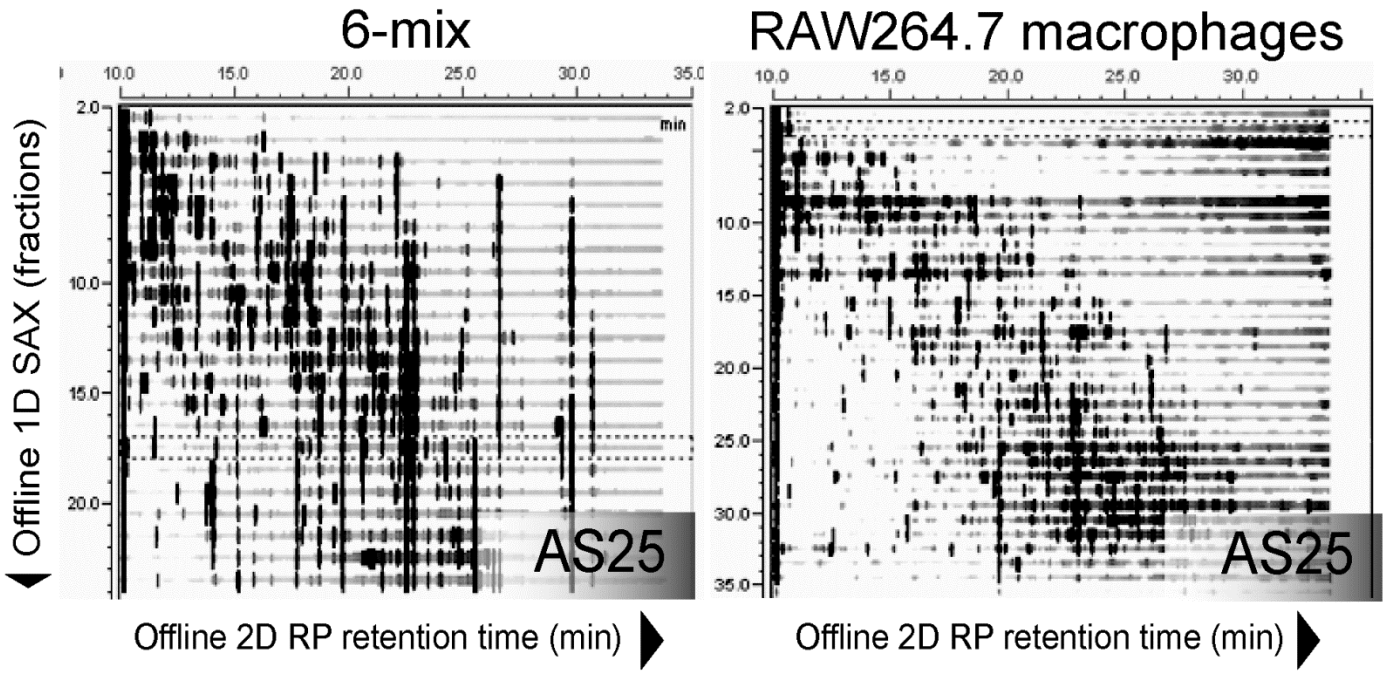
- "*Table S-1_**x**_TPP*"= 3 tables, which contain information about identified proteins (iProphet/ProteinProphet, FDR=1%) and identified peptides (Peptide Prophet, FDR=1% at peptide level). **x**= SAX or RP or SAXRP

- "*Table S-1_**y**_MQ*"= 3 tables, which contain information about identified proteins peptides by MaxQuant (MQ). **y**= SAX or RP or SAXRP

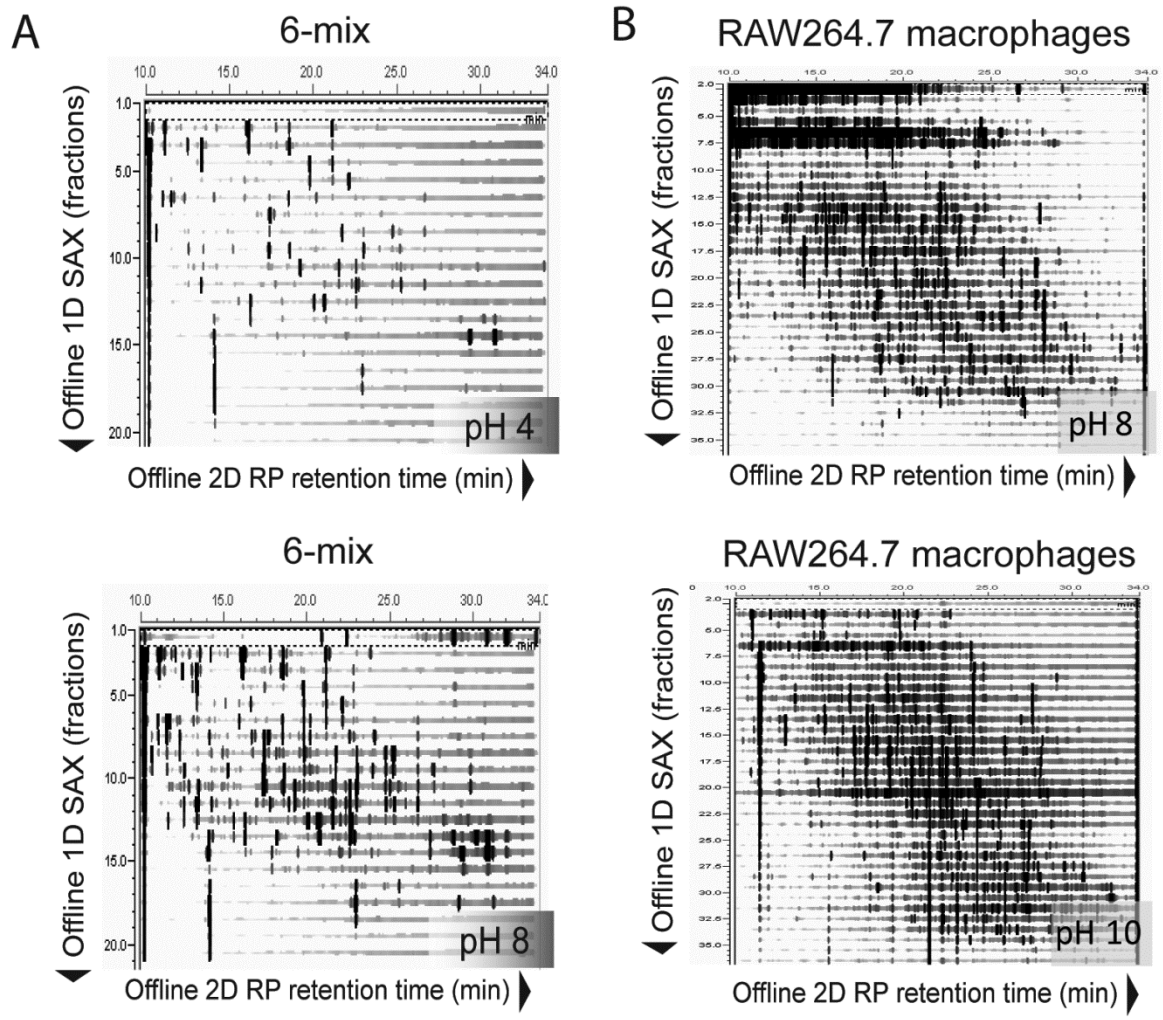
Supporting Information, Figure S1



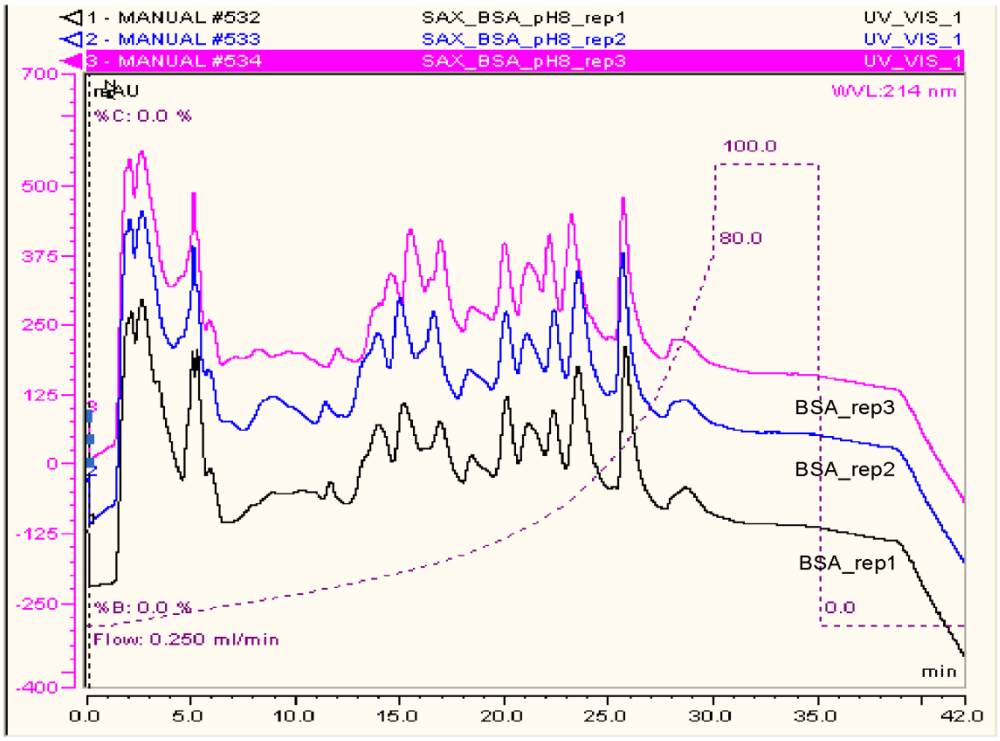
Supporting Information, Figure S2



Supporting Information, Figure S3



Supporting Information, Figure S4



Sample	Peak Width	Resol.	Skewness	Plates	Zero Moment	First Moment	Second Moment
	min	(EP)		(EP)	mAU*min	min	min²
BSA_rep1	1.0181	1.5520	1.3041	5026.0588	50.3479	14.2489	0.0476
BSA_rep2	1.0613	1.5308	1.3392	5284.9333	59.7326	14.8118	0.0566
BSA_rep3	1.2829	1.7243	1.3359	5191.7647	52.3908	14.6605	0.0456

Table legend

Peak width (min): peak width extrapolated on the baseline

Resol. (EP): measure of the separation between two peaks. This resolution helps to evaluate the separation capability of the column.
EP: European Pharmacopeia

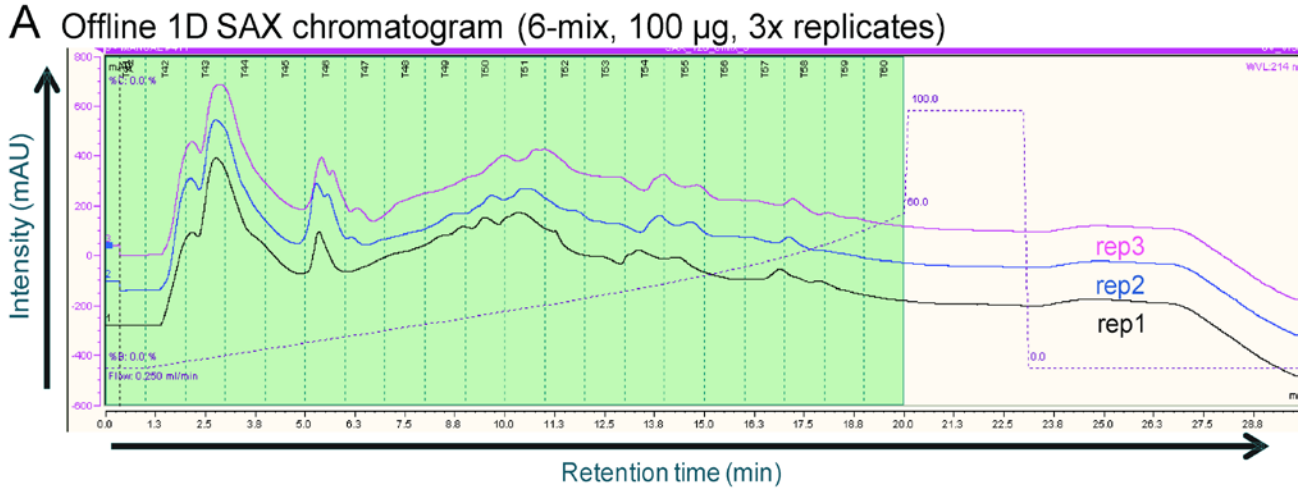
Skewness: measure for the column quality (similar to asymmetry).

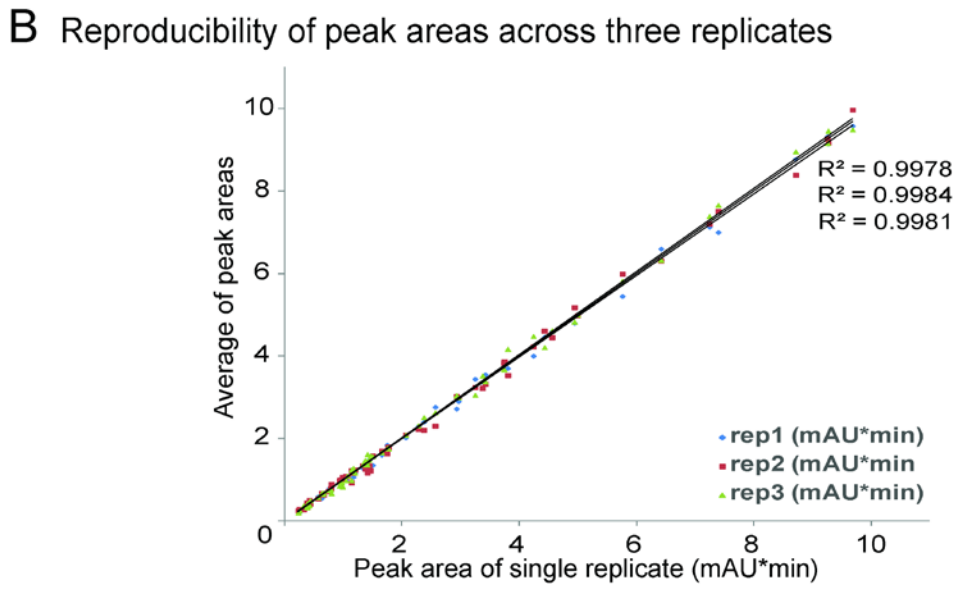
Plates (EP): measure of the separation capability of the column.
EP: European Pharmacopeia

Statistical moments: describe the chromatographic separation much better than the values for the theoretical plates and resolution.
This is because statistical moments are not based upon an ideal Gaussian distribution, but on the actual statistical distribution of data points.

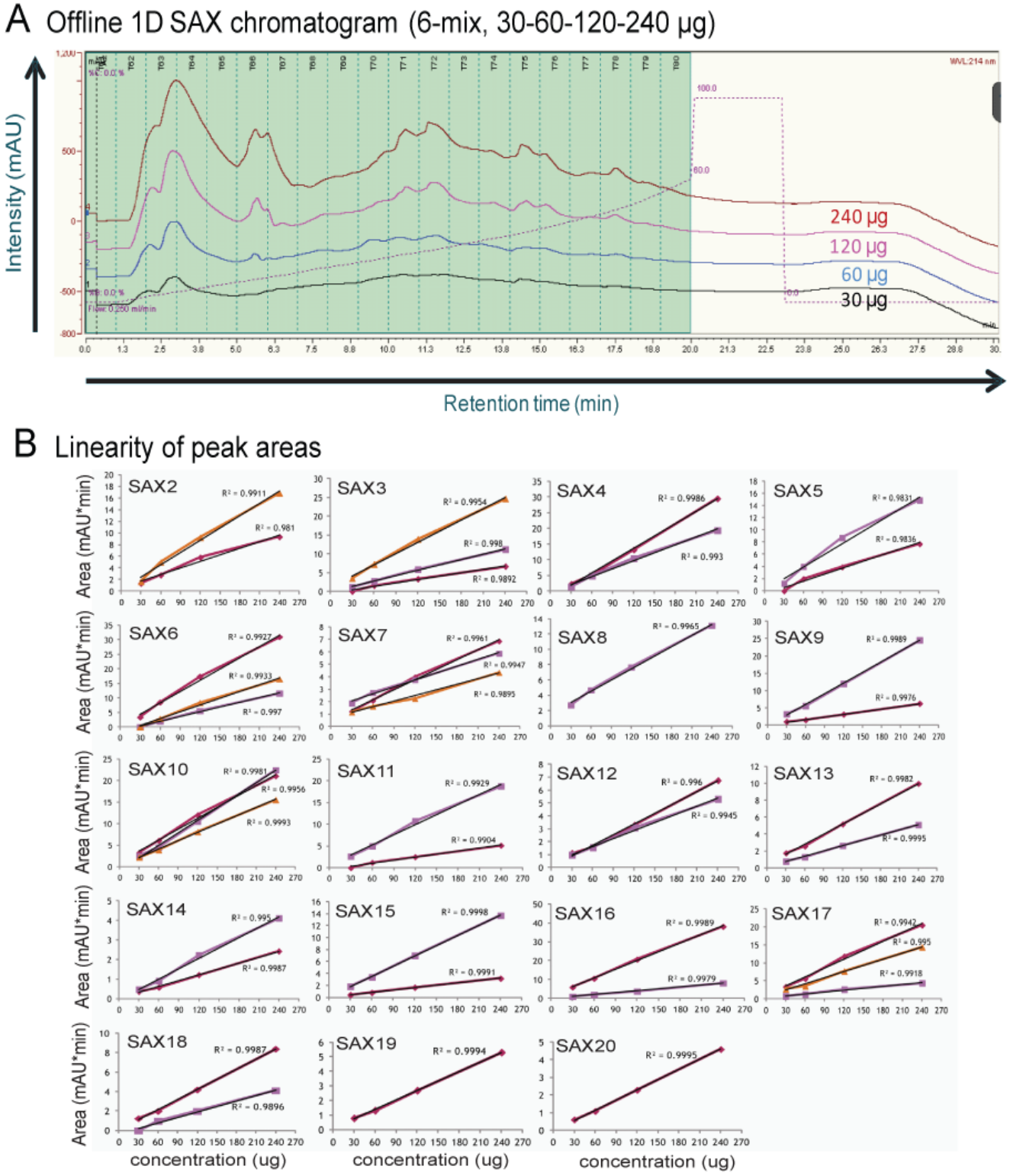
Zero moment = peak area
First moment = averaged retention time
Second moment = retention time variance
(the narrower the peak, the smaller the variance).

Supporting Information, Figure S5

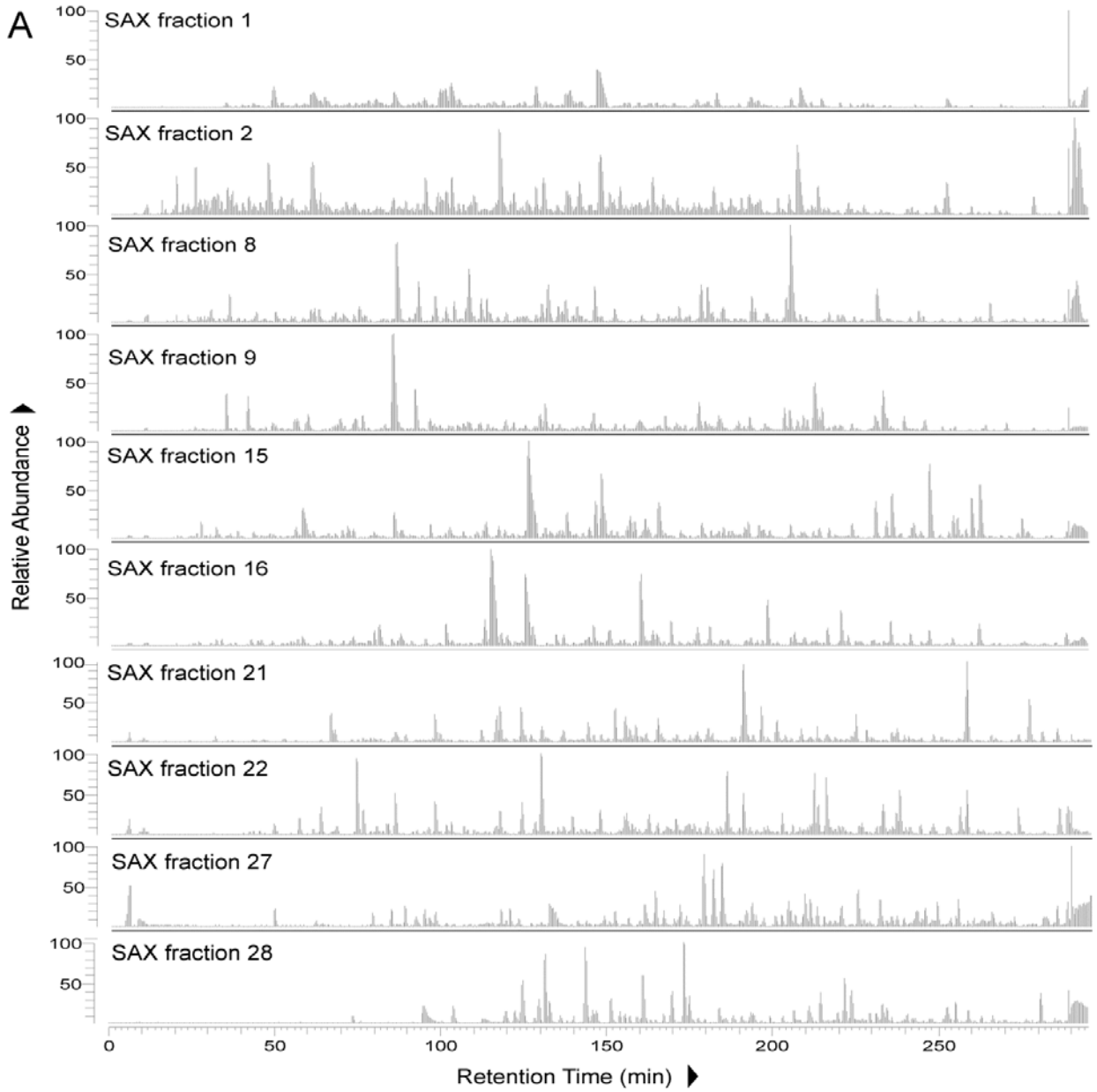




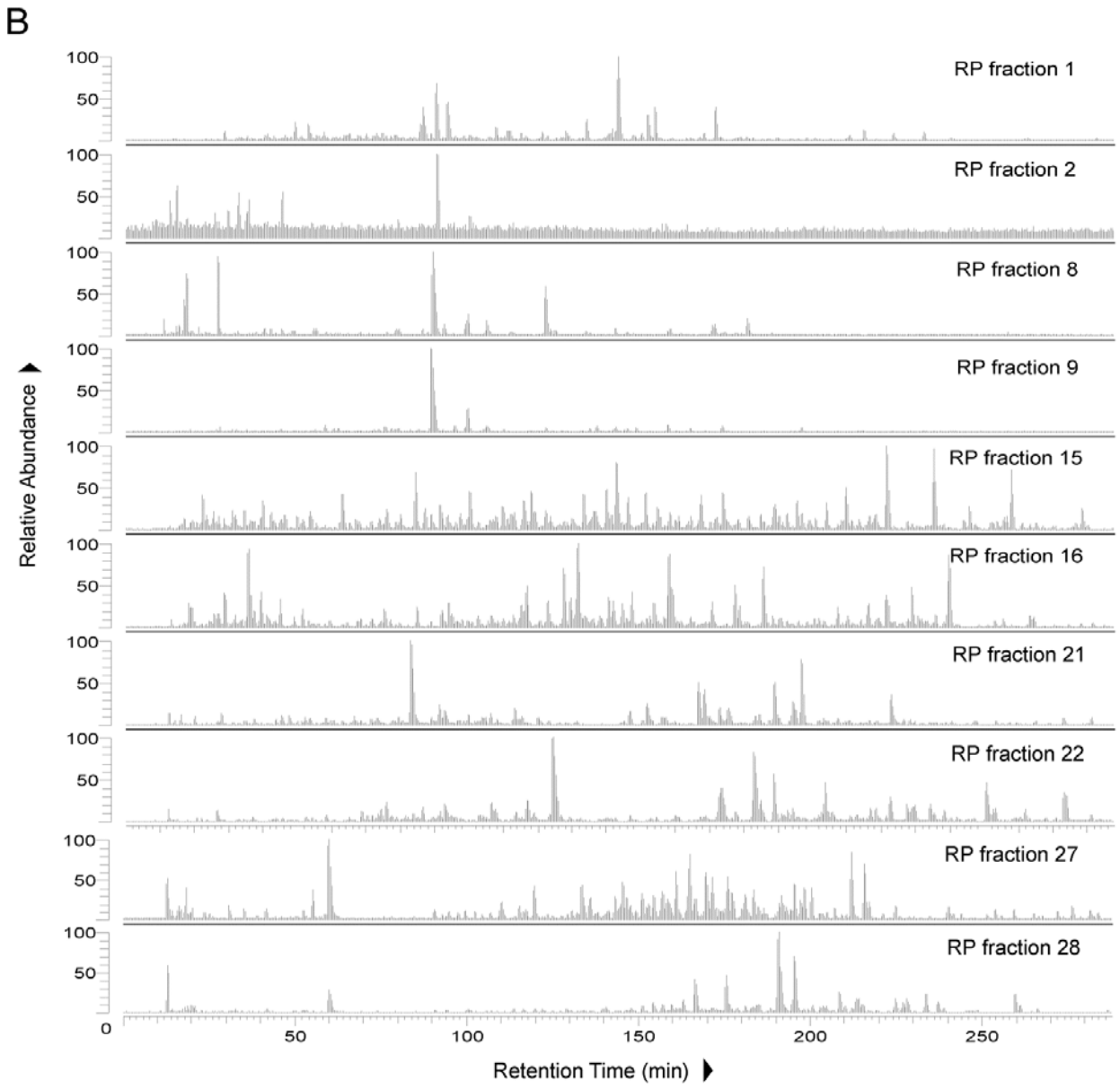
Supporting Information, Figure S6



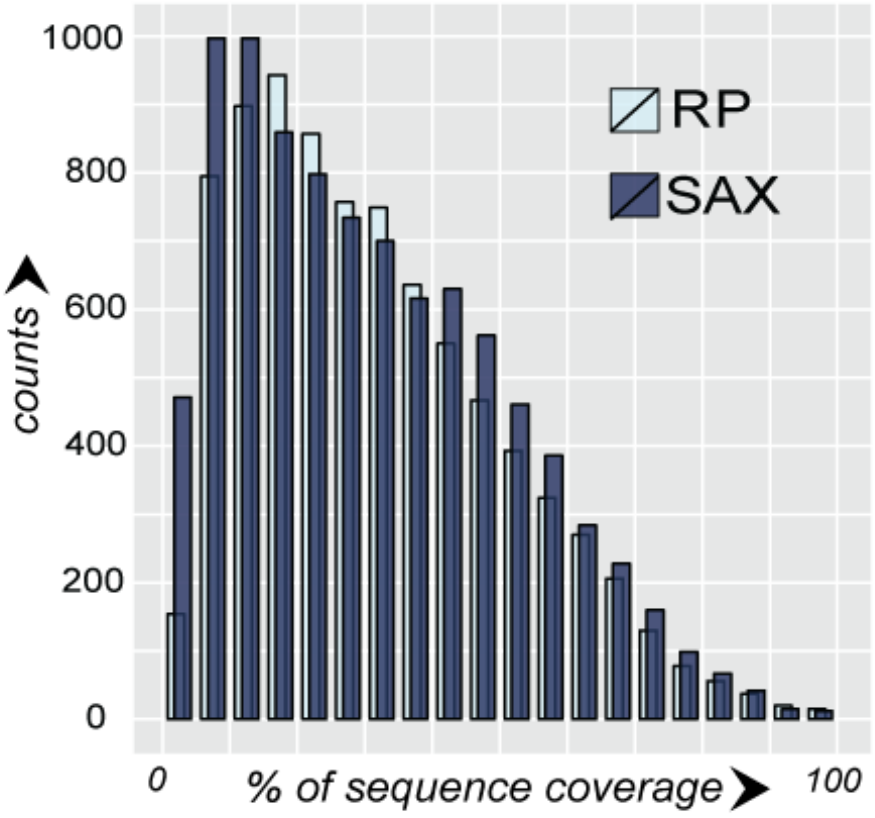
Supporting Information, Figure S7 (A)



Supporting Information, Figure S7 (B)



Supporting Information, Figure S8



AVERAGE PROTEIN SEQUENCE COVERAGE SAX = 31%

AVERAGE PROTEIN SEQUENCE COVERAGE RP = 32%

AVERAGE PROTEIN SEQUENCE COVERAGE SAX/RP = 38%

(*Based on "Table S-1_x_TPP"*)

Supporting Information, Figure S9